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APPLICATION NUMBER: *60/526,786*
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RELATED PCT APPLICATION NUMBER: *PCT/US04/40825*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.53(c).

	Docket Number	14014.0417U1	Type a Plus Sign (+) inside this box	+

INVENTOR(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)
Chiorini	John	A.	2604 Loma St., Silver Spring, MD 20902 U.S. Citizen

TITLE OF INVENTION (500 characters max)

BOVINE ADENO-ASSOCIATED VIRAL (BAAV) VECTOR AND USES THEREOF

CORRESPONDENCE ADDRESS

Customer Number 23859

ENCLOSED APPLICATION PARTS (*Check All That Apply*)

- ☒ Provisional Application Title Page — *Number of Pages* [1]
- ☒ Specification (includes Description, Claims, & Abstract) — *Number of Pages* [74]
- ☒ Drawing(s) — *Number of Sheets* [25]
- ☒ Authorization to Treat Reply Requesting Extension of Time as Incorporating Petition for Extension of Time
- ☒ Other (specify): Return Postcard

METHOD PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*Check One*)	
☐ Applicant claims small entity status. See 37 CFR § 1.27. ☒ A Credit Card Payment Form PTO-2038 is enclosed to cover the filing fees. ☐ A check or money order is enclosed to cover the filing fees. ☐ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number _____. ☒ The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 14-0629.	**FILING FEE AMOUNT** $ 160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

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Respectfully submitted,

Signature _____ **Date** 12/4/03

Typed or Printed Name: Lizette M. Fernandez, Ph.D.

Registration No. 46,694

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

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_____ 12/4/03

Michael Laird — Date

ATTORNEY DOCKET NO. 14014.0417U1
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
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Chiorini, John A.	)	Art Unit: Unassigned
	)	
Application No. Unassigned	)	Examiner: Unassigned
	)	
Filing Date: Concurrently	)	Confirmation No. Unassigned
	)	
For: **BOVINE ADENO-ASSOCIATED VIRAL (BAAV) VECTOR AND USES THEREOF**	)	

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Pursuant to 37 C.F.R. § 1.136(a)(3), the Commissioner is hereby requested and authorized to treat any concurrent or future reply in the above-identified application, requiring a petition for an extension of time for its timely submission, as incorporating a petition for extension of time for the appropriate length of time.

ATTORNEY DOCKET NO. 14014.0417U1
PATENT

The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

NEEDLE & ROSENBERG, P.C.

Lizette M. Fernandez, Ph.D.
Registration No. 46,694

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Express Mail No. EL 992 075 554 US

Attorney Docket No. 14014.0417U1

UTILITY PATENT - PROVISIONAL FILING

PROVISIONAL APPLICATION FOR LETTERS PATENT

TO ALL WHOM IT MAY CONCERN:

Be it known that I, JOHN A. CHIORINI, residing at 2604 Loma St., Silver Spring, MD 20902, U.S.A., have invented new and useful improvements in

BOVINE ADENO-ASSOCIATED VIRAL (BAAV) VECTOR AND USES THEREOF

for which the following is a specification.

ATTORNEY DOCKET NO. 14014.0417U1

BOVINE ADENO-ASSOCIATED VIRAL (BAAV) VECTOR AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention provides bovine adeno-associated virus (BAAV) and vectors derived therefrom. Thus, the present invention relates to BAAV vectors for and methods of delivering nucleic acids to cells of subjects.

Background Art

Adeno-associated virus (AAV) is a member of the Parvoviridae, a virus family characterized by a single stranded linear DNA genome and a small icosahedral shaped capsid measuring about 20nm in diameter. AAV was first described as a contamination of tissue culture grown simian virus 15, a simian adeno virus and was found dependent on adenovirus for measurable replication. This lead to its name, adeno- associated virus, and its classification in the genus Dependovirus (reviewed in (Hoggan, 1970)). AAV is a common contaminant of adenovirus samples and has been isolated from human virus samples (AAV-2, AAV-3, AAV-5), from samples of simian virus-15 infected cells (AAV-1, AAV-4) as well as from stocks of avian (AAAV) (Bossis and Chiorini, 2003)) bovine, canine and ovine adenovirus and laboratory adenovirus type 5 stock (AAV-6). DNA spanning the entire rep-cap ORFs of AAV7 and AAV8 was amplified by PCR from heart tissue of rhesus monkeys (Gao et al., 2002) .
With the exception of AAVs 1 and 6 all cloned AAV isolates appear to be serologically distinct. Eight isolates have been cloned, and recombinant viral stocks have been generated from each isolated.

AAV appears to be a common infection in humans. 50%-80% of adults in North America are seropositive for AAV. A steep rise in antibody response against AAV 1-3 was observed in the age group between 1-10 years (Blacklow et al., 1968). AAV is readily isolated from anal and throat specimens from children (Blacklow et al., 1967) whereas isolation from adults was not observed. It appears that AAV spreads primarily

in the young population (Hoggan, 1970). Prevalence of antibodies against AAV was found to be similar in Europe, Brazil and Japan, which suggests a global spread of AAV (Erles et al., 1999). Infection with AAV appears to be benign in man and laboratory animals. Currently, no disease has been associated with AAV infections. AAV-2 is the best characterized adeno-associated virus and will be discussed as an AAV prototype. The AAV-2 genome consists of a linear single stranded DNA of 4.780 nucleotides. Both polarities of DNA are encapsulated by AAV with equal efficiency. The AAV genome contains 2 open reading frames named rep and cap. The rep ORF encodes the non-structural proteins witch are essential for viral DNA replication, packaging as well as AAV integration. The cap ORF encodes the capsid proteins. The rep ORF is transcribed from promoters at map units P5 and P19. The rep transcripts contain an intron close to the 3' end of the rep ORF and can be alternatively spliced. The rep ORF is therefore expressed as 4 partially overlapping proteins, which were termed according to their molecular weight Rep78, 68, 52 and 40. The cap ORF is expressed from a single promoter at P40. By alternative splicing and utilization of an alternative ACG start codon, cap is expressed into the capsid proteins VP 1-3 which range in size from 65-86 kDa. VP 3 is the most abundant capsid protein and constitutes 80% of the capsid. All viral transcripts terminate at a polyA signal at map unit 96. During a productive AAV infection, unspliced mRNA from the p5 promoter encoding Rep78 are the first detectable viral transcripts. In the course of infection, expression from P5, P19 and P40 increase to 1:3:18 levels respectively. The levels of spliced transcripts increased to 50% for P5, P19 products and 90% of P40 expressed RNA (Mouw and Pintel, 2000).

The genome is terminated on both sides by inverted terminal repeats (ITRs) of 145 nucleotides (nt). 125 nt of the ITR constitute a palindrome which contains 2 internal palindroms of 21 nt each. The ITR can fold back on itself to generate a T-shaped hairpin with only 7 non-paired bases. The stem of the ITR contains a Rep binding site (RBS) and a sequence that is site and strand specifically cleaved by Rep – the terminal resolution site (TRS). The ITR is essential for AAV genome replication, integration and contains the packaging signals.

The single-stranded AAV genome is packaged into a non-enveloped icosahedral shaped capsid of about 20-25 nm diameter. The virion consists of 26% DNA and 74% protein and has a density of 1.41 g/cm^3. AAV particles are extremely stable and can withstand heating to 60°C for 1 hour, extreme ph, and extraction with organic solvents.

Rep proteins are involved in almost every step of AAV replication including AAV genome replication, integration, and packaging. Rep78 and Rep68 possess ATPase, 3'-5' helicase, ligase and nicking activities and bind specifically to DNA. Rep52 and Rep40 appear to be involved in the encapsidation process and encode ATPase and 3'-5' helicase activities. Mutational analysis suggests a domain structure for Rep78. The N-terminal 225 aa are involved in DNA binding, DNA nicking and ligation. Rep78 and Rep68 recognize a GCTC repeat motif in the ITR as well as in a linear truncated form of the ITR (Chiorini et al., 1994) with similar efficiencies. Rep78 and Rep68 possess a sequence and strand specific endonuclease activity, which cleaves the ITR at the terminal resolution site (TRS). Rep endonuclease activity is dependent on nucleoside triphosphate hydrolysis and presence of metal cations. Rep 78 and 68 can also bind and cleave single stranded DNA in a NTP independent matter. In addition Rep78 catalyzes rejoining of single stranded DNA substrates originating from the AAV origin of replication – i.e. sequences containing a rep binding and terminal resolution element.

The central region of Rep78, which represents the N-terminus of Rep52 and Rep40, contains the ATPase and 3'-5' helicase activities as well as nuclear localization signals. The helicase activity unwinds DNA-DNA and DNA-RNA duplexes, but not RNA-RNA. The ATPase activity is constitutive and independent of a DNA substrate. The c-terminus of Rep78 contains a potential zinc-finger domain and can inhibit the cellular serine/ theonine kinase activity of PKA as well as its homolog PRKX by pseudosubstrate inhibition. Rep68 which is translated from a spliced mRNA that encodes the N-terminal 529 amino acids (aa) of Rep78 fused to 7 aa unique for Rep68, doesn't inhibit either PKA or PRKX. In addition to these biochemical activities, Rep can affect intracellular conditions by protein-protein interactions. Rep78 binds to a variety of cellular proteins including transcription factors like SP-1, high-mobility-

group non-histone protein 1 (HMG-1) and the oncosuppressor p53. Overexpression of Rep results in pleiotrophic effects. Rep78 disrupts cell cycle progression and inhibits transformation by cellular and viral oncogens. In susceptible cell lines, overexpression of Rep resulted in apoptosis and cell death. Several of Rep78 activities contribute to cytotxicity, including its constitutive ATPase activity , interference with cellular gene expression and protein interactions.

The first step of an AAV infection is binding to the cell surface. Receptors and coreceptors for AAV2 include heparan sulfate proteoglycan, fibroblast growth factor receptor-1, and αvb5 integrins whereas N-linked 2,3-linked sialic acid is required for AAV-5 binding and transduction (Walters et al., 2001). In Hela cells, fluorescently labeled AAV2 particles appear to enter the cell via receptor-mediated endocytosis in clathrin coated pits. More than 60% of bound virus was internalized within 10 min after infection. Labeled AAV particles are observed to have escaped from the endosome and trafficked via the cytoplasm to the cell nucleus and accumulated perinuclear, before entering the nucleus, probably via nuclear pore complex (NPC). AAV2 particles have been detected in the nucleus, suggesting that uncoating takes place in the nucleus (Bartlett et al., 2000; Sanlioglu et al., 2000). AAV-5 is internalized in Hela cells predominantly by clathrin coated vesicles but to a lesser degree also in noncoated pits. AAV particles trafficked intercellular via the Golgi apparatus (Bantel-Schaal et al., 2002). At least partial uncoating of AAV5 was suggested to take place before entering the nucleus since intact AAV5 particles could not be detected in the nucleus (Bantel-Schaal et al., 2002) After uncoating, the single stranded genome is converted into duplex DNA either by leading strand synthesis or annealing of input DNA of opposite polarity. AAV replication takes place within the nucleus.

During a coinfection with a helper virus such as Adenovirus, herpes simplex virus or cytomegalovirus, AAV is capable of an efficient productive replication. The helper functions provided by Adenovirus have been studied in great detail. In human embryonic kidney 293 cells, which constitutively express the Adenovirus E1A and E1B genes, the early Ad gene products of E2A, E4 and VA were found sufficient to allow replication of recombinant AAV. Allen et al. reported that efficient production of rAAV is possible in 293 cells transfected with only an E4orf6 expression plasmid

(Allen et al., 2000). E1A stimulates S phase entry and induces unscheduled DNA synthesis by inactivating the pRB checkpoint at the G1/S border by interaction with pRB family proteins which results in the release of E2F (reviewed in (Ben-Israel and Kleinberger, 2002). This leads to either induction or activation of enzymes involved in nucleotide synthesis and DNA replication. Since unscheduled DNA synthesis is a strong apoptotic signal, anti-apoptotic functions are required. E1B-19k is a Bcl-2 homolog and E1B-55k is a p53 antagonist. Both proteins have anti-apoptotic functions. E4orf6 forms a complex with E1B-55k and results in degradation of p53. It is also reported to cause S-phase arrest (Ben-Israel and Kleinberger, 2002). E2A encodes a single strand DNA binding protein, which appears to be non-essential for DNA replication but effects gene expression (Chang and Shenk, 1990) (Fields 39, 40). The VA transcription unit effects AAV RNA stability and translation (Janik et al., 1989). E1A has a more direct effect on AAV gene expression. The cellular transcription factor YY-1 binds and inhibits the viral P5 promoter. E1A relieves this transcriptional block. None of the late Ad gene products have been found to be essential for AAV replication. The main function of the helper virus appears to be the generation of a cellular environment with active DNA replication machinery and blocked pro-apoptotic functions, that allows high-level AAV replication rather than a direct involvement in AAV replication.

While AAV is usually dependent on a helper virus for efficient replication, low level AAV replication was observed under conditions of genotoxic stress (Yakinoglu et al., 1988; Yakobson et al., 1989). AAV DNA replication and particle formation was also observed in differentiating keratinocytes in the absence of helper virus infection (Meyers et al., 2000). This demonstrates that AAV is not defective per se but rather depends on the helper virus to establish the favorable cellular condition and to provide factors for efficient replication

~~The~~ ability ~~of~~ AAV ~~vectors to infect~~ dividing ~~and non-dividing~~ cells, establish long-term transgene expression, and the lack of pathogenicity has made them attractive for use in gene therapy applications. Lack of cross competition in binding experiments suggests that each AAV serotype may have a distinct mechanism of cell entry. Comparison of the *cap* ORFs from different serotypes has identified blocks of

conserved and divergent sequence, with most of the later residing on the exterior of the virion, thus explaining the altered tissue tropism among serotypes (19-21, 48, 56). Vectors based on new AAV serotypes may have different host range and different immunological properties, thus allowing for more efficient transduction in certain cell types. In addition, characterization of new serotypes will aid in identifying viral elements required for altered tissue tropism.

Adeno-associated virus has been detected in strains of bovine adenovirus (BAV) type I and type II. It was isolated from the lysate of BAV infected cells by CsCl gradient centrifugation and is described to band at densities between 1.37-1.38 (Luchsinger et al., 1971) or 1.38-1.39 g/cm^3 (Myrup et al., 1976). The bovine AAV isolated from BAV I was found to be serological distinct from AAV1-4 (Luchsinger et al., 1970) while AAV isolated from BAV II was serological indistinquishable from AAV contaminations in BAV I (Myrup et al., 1976). The size of the bovine AAV (BAAV) was estimated by electron microscopy to be about 220 Å in diameter.

BAAV can be a useful tool for gene transfer, since BAAV is serologically distinct from primate AAVs and may therefore not be neutralized from antibodies generated in humans during human AAV infections or gene transfer using primate AAV vectors. In addition, new AAV serotypes may use different receptors for cell entry and have therefore a distinct tropism, which results in the most efficient transduction in certain cell types.

The present invention provides the isolation, subcloning, and sequencing of BAAV from Bovine Adenovirus Type 1 (ATCC VR-313) and Bovine Adenovirus Type 2 (ATCC VR-314) obtained from ATCC, which are reported by ATCC to be contaminated with AAV. A BAAV packaging plasmid was made and a recombinant BAAV was generated to study the tropism of BAAV as well as to study its feasibility as a vector for in vivo gene transfer.

The genome of BAAV is [redacted] nucleotides in length and has similar organization with that of other AAVs. The entire genome of BAAV displays 54-79% identity at the nucleotide level with the other known AAVs. Highest homology was observed with AAV5 (79%), and the lowest with AAAV (54%) The BAAV genome has inverted terminal repeats of 150 nucleotides and form a characteristic T-shaped

palindromic structure. The putative Rep-binding element (RBE) consists of a tandem $(GAGY)_4$ repeat, and the putative terminal resolution site (*trs*), AGTGTGG. Surprisingly the BAAV ITR is greater than 95% identical to AAV5 and contains a trs, which is identical to the AAV5 trs as well a conserved RRE. The Rep ORF of BAAV displays 48-89 % identity at the amino acid level with the other AAVs, with most of the diversity clustered at the amino termini. A surprisingly high homology of 89 % was found with AAV5. Comparison of the capsid proteins of BAAV and other dependoviruses revealed 55-76 % identity in VP1. AAV4 showed the highest homology to BAAV with 76% while AAAV was most divergent with 55% identity to BAAV VP1. Divergent regions in the capsid ORF are clustered in surface exposed loops. Due to the high homology between the BAAV and AAV5 ITR as well as Rep sequence, it was hypothesized that recombinant BAAV particles carrying a *lacZ* reporter gene or a GFP expression cassette could be produced by co-transfection of AAV5 ITR containing vector plasmids with BAAV packaging and an adenovirus helper plasmids in 293T cells. The recombinant particles have a buoyant density in CsCl gradients of 1.375 gm/cm^3 which is similar to AAV4. These recombinant particles have been used to compare the transduction efficiency of BAAV with other know AAV isolates and it was found that BAAV has a unique transduction profile compared to other isolates and is able to transduce a wide variety of tumor cells including cells of CNS, colon, prostate, renal, breast and ovarian lineage.

The present invention provides a vector comprising the BAAV virus or a vector comprising subparts of the virus, as well as BAAV viral particles. While BAAV is similar to AAV1-8, the viruses are found herein to be physically and genetically distinct. These differences endow BAAV with some unique properties and advantages, which better suit it as a vector for gene therapy or gene transfer applications. As shown herein, BAAV capsid proteins are distinct from primate and avian AAV capsid proteins and exhibits a distinct cell tropism, thus making BAAV capsid-containing particles suitable for transducing cell types for which primate or avian recombinant AAV particles are unsuited or less well-suited. BAAV is serologically distinct from other AAVs and humans are not reported to have neutralizing antibodies against BAAV, thus in a gene therapy application, BAAV would allow for transduction

of a patient who already possesses neutralizing antibodies to primate isolates either as a result of natural immunological defense or from prior exposure to other vectors. Thus, the present invention, by providing these new recombinant vectors and particles based on BAAV provides a new and highly useful series of vectors.

SUMMARY OF THE INVENTION

The present invention provides a nucleic acid vector comprising a pair of bovine adeno-associated virus (BAAV) inverted terminal repeats and a promoter between the inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV1 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV2 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV3 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV4 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV5 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV6 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV7 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV8 inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAAV inverted terminal repeats.

The present invention further provides an BAAV particle containing a vector comprising a pair of AAV5 inverted terminal repeats.

The present invention further provides an AAV1 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV2 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV3 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV4 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV5 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV6 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV7 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAV8 particle containing a vector comprising a pair of BAAV inverted terminal repeats.

The present invention further provides an AAAV particle containing a vector comprising a pair of BAAV inverted terminal repeats

The present invention further provides a dependovirus particle containing a vector comprising a pair of BAAV inverted terminal repeats

Additionally, the instant invention provides an isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:1 (BAAV genome). Furthermore, the present invention provides an isolated nucleic acid consisting essentially of the nucleotide sequence set forth in SEQ ID NO:1 (BAAV genome).

The present invention provides an isolated nucleic acid encoding an BAAV Rep78 protein, for example, the nucleic acid as set forth in SEQ ID NO:2. Additionally provided is an isolated full-length BAAV Rep78 protein as set forth in SEQ ID NO:3 or

a unique fragment thereof. Additionally, provided is an isolated BAAV Rep 52 protein encoded by nucleic acid as set forth in SEQ ID NO:4 having the amino acid sequence set forth in SEQ ID NO:5, or a unique fragment thereof. The sequences for these proteins as well as the nucleotide sequence of the corresponding open reading frames are provided below in the Sequence Listing and elsewhere in the application where the proteins are described.

The present invention further provides an isolated BAAV capsid protein, VP1, encoded by nucleic acid as set forth in SEQ ID NO:6 having the amino acid sequence set forth in SEQ ID NO:7, or a unique fragment thereof. Additionally provided is an isolated BAAV capsid protein, VP2, encoded by nucleic acid as set forth in SEQ ID NO:8 having the amino acid sequence set forth in SEQ ID NO:9, or a unique fragment thereof. Also provided is an isolated BAAV capsid protein, VP3, encoded by nucleic acid as set forth in SEQ ID NO:10 having the amino acid sequence set forth in SEQ ID NO:11, or a unique fragment thereof.

Additionally provided by the present invention is an isolated nucleic acid comprising an BAAV p5 promoter having the nucleic acid sequence set forth in SEQ ID NO: 15, or a unique fragment thereof.

The instant invention provides a method of screening a cell for infectivity by BAAV comprising contacting the cell with BAAV and detecting the presence of BAAV in the cells.

The present invention further provides a method of delivering a nucleic acid to a cell comprising administering to the cell an BAAV particle containing a vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to the cell.

The present invention also provides a method of delivering a nucleic acid to a subject comprising administering to a cell from the subject an BAAV particle comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, and returning the cell to the subject, thereby delivering the nucleic acid to the subject.

The present invention also provides a method of delivering a nucleic acid to a cell in a subject comprising administering to the subject an BAAV particle comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby

delivering the nucleic acid to a cell in the subject.

The instant invention further provides a method of delivering a nucleic acid to a cell in a subject having antibodies to other serotypes of AAV comprising administering to the subject an BAAV particle comprising the nucleic acid, thereby delivering the nucleic acid to a cell in the subject.

The present invention further provides a BAAV particle comprising a capsid protein consisting essentially of the amino acid sequence set forth in SEQ ID NO:7, or a unique fragment thereof.

Additionally provided by the present invention is an isolated nucleic acid comprising an BAAV p5 promoter having the nucleic acid sequence set forth in SEQ ID NO:15, or a unique fragment thereof.

The instant invention provides a method of screening a cell for infectivity by BAAV comprising contacting the cell with BAAV and detecting the presence of BAAV in the cells.

The present invention further provides a method of delivering a nucleic acid to a cell comprising administering to the cell an BAAV particle containing a vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to the cell.

The present invention also provides a method of delivering a nucleic acid to a subject comprising administering to a cell from the subject an BAAV particle comprising the nucleic acid inserted between a pair of BAAV inverted terminal repeats, and returning the cell to the subject, thereby delivering the nucleic acid to the subject.

The present invention also provides a method of delivering a nucleic acid to a cell in a subject comprising administering to the subject an BAAV particle comprising the nucleic acid inserted between a pair of BAAV inverted terminal repeats, thereby ~~delivering the nucleic acid to a cell in the~~ subject.

The instant invention further provides a method of delivering a nucleic acid to a cell in a subject having antibodies to primate AAVs comprising administering to the subject an BAAV particle comprising the nucleic acid, thereby delivering the nucleic acid to a cell in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an example of the BAAV genome. (A) The genomes of BAAV, AAV2, AAV4 and AAV5 were aligned using MacVector (Oxford Molecular). Nucleotides identical in at least 2 AAV serotypes are displayed boxed and shaded. (B) Phylogenetic relationship of BAAV to other AAV serotypes is illustrated by an unrooted tree diagram.

Figure 2 shows an example of a BAAV ITR. The sequence of the ITR is shown in hairpin confirmation. The putative Rep binding site and TRS element are boxed. Sequence changes relative to the AAV5 ITR are annotated either above or below the BAAV sequence in bold letters.

Figure 3 illustrated comparisons of Rep and Vp1 amino acid sequences. The (A) rep and (B) cap ORFs of BAAV were aligned to the corresponding amino acid sequences of AAV2, AAV5 and AAV2, AAV respectively using MacVector. Identical amino acids are indicated by a dark shaded box, similar amino acids by a light shaded box. Dashes indicate gaps in the sequence added by the alignment program. Phylogenetic relationship of (C) BAAV Rep and (D) Vp1 to other AAV serotypes is illustrated by an unrooted tree diagram.

Figure 4 shows the transduction profile of BAAV in 60 cancer cell lines. Human cell lines were infected with rBAAV expressing lacZ in serial dilutions and coinfected with a MOI of 10 with Ad5. Columns represent beta-Gal transducing units/ 10^9 DNAse resistant rAAV particles.

Figure 5 shows that BAAV elicits a distinct immune response in mice. rBAAVlacZ (A) and rAAV4lacZ (B) were incubated with serial dilutions of polyclonal mice serum against rAAV2, rAAV4, rAAV5 and rBAAV. Cos cells coinfected with

Ad5 (MOI = 10) were incubated with the virus/serum mixture. % neutralization was calculated by the formula: 100x (1- transducing titers of serum incubated rAAV/ untreated rAAV). Values of neutralization that were calculated to be below zero were adjusted to zero. Values given are means of 3 experiments, error bars represent standard deviation. BAAV transduction efficiency was unaffected by antisera against AAV2, AAV4 and AAV5. Antisera against BAAV blocked infection of BAAV but had no effect on the other AAV serotypes.

Figure 6 is a comparison of rAAV2 and rBAAV transduction of salivary glands. 10^{10} particles of AAV2-RnlacZ and BAAV-RnlacZ were injected into submandibular glands of BALB/c mice by retrograde ductal instillation. 4 weeks after infection, glands were removed and analyzed for the presence of vector genome DNA by real time PCR (A) and expression of beta-gal by an ELISA (B). Values given are means of data from 7 animals, error bars represent standard deviation.

DETAILED DESCRIPTION OF THE INVENTION

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used. The terms "having" and "comprising" are used interchangeably herein, and signify open ended meaning.

The present application provides a recombinant bovine adeno-associated virus (BAAV). This virus has one or more of the characteristics described below. The compositions of the present invention do not include wild-type BAAV. The methods of the present invention can use either wild-type BAAV or recombinant BAAV-based delivery.

The present invention provides novel BAAV particles, recombinant BAAV vectors and recombinant BAAV virions. An BAAV particle is a viral particle comprising an BAAV capsid protein. A recombinant BAAV vector is a nucleic acid construct that comprises at least one unique nucleic acid of BAAV. A recombinant BAAV virion is a particle containing a recombinant BAAV vector, wherin the particle can be either an BAAV particle as described herein or a non-BAAV particle. Alternatively, the recombinant BAAV virion is an BAAV particle containing a recombinant vector, wherein the vector can be either an BAAV vector as described herein or a non-BAAV vector. These vectors, particles, virions, nucleic acids and polypeptides are described below.

The present invention provides the nucleotide sequence of the BAAV genome and vectors and particles derived therefrom. Specifically, the present invention provides a nucleic acid vector comprising a pair of BAAV inverted terminal repeats (ITRs) and a promoter between the inverted terminal repeats. The rep proteins of AAV5 and BAAV will bind to the BAAV ITR and it can function as an origin of replication for packaging of recombinant AAV particles. The minimum sequence necessary for this activity is the TRS site where Rep cleaves in order to replicate the virus. Minor modifications in an ITR are contemplated and are those that will not interfere with the hairpin structure formed by the ITR as described herein and known in the art. Furthermore, to be considered within the term e.g. it must retain the Rep binding site described herein.

The D- region of the AAV2 ITR, a single stranded region of the ITR, inboard of the TRS site, has been shown to bind a factor which depending on its phosphorylation state correlates with the conversion of the AAV from a single stranded genome to a transcriptionally active form that allows for expression of the viral DNA. This region is conserved between AAV2, 3, 4, and 6 but is divergent in AAV5 and BAAV (SEQ ID NO: 13). The D+ region is the reverse complement of the D- region.

The promoter can be any desired promoter, selected by known considerations, such as the level of expression of a nucleic acid functionally linked to the promoter and

the cell type in which the vector is to be used. That is, the promoter can be tissue/cell-specific. Promoters can be prokaryotic, eukaryotic, fungal, nuclear, mitochondrial, viral or plant promoters. Promoters can be exogenous or endogenous to the cell type being transduced by the vector. Promoters can include, for example, bacterial promoters, known strong promoters such as SV40 or the inducible metallothionein promoter, or an AAV promoter, such as an AAV p5 promoter. Additionally, chimeric regulatory promoters for targeted gene expression can be utilized. Examples of these regulatory systems, which are known in the art, include the tetracycline based regulatory system which utilizes the tet transactivator protein (tTA), a chimeric protein containing the VP16 activation domain fused to the tet repressor of *Escherichia coli,* the IPTG based regulatory system, the CID based regulatory system, and the Ecdysone based regulatory system (44). Other promoters include promoters derived from actin genes, immunoglobulin genes, cytomegalovirus (CMV), adenovirus, bovine papilloma virus, adenoviral promoters, such as the adenoviral major late promoter, an inducible heat shock promoter, respiratory syncytial virus, Rous sarcomas virus (RSV), etc., specifically, the promoter can be AAV2 p5 promoter or AAV5 p5 promoter or BAAV p5 promoter. More specifically, the BAAV p5 promoter can be in about the same location in SEQ ID NO: 1 as the AAV2 p5 promoter, in the corresponding AAV2 published sequence. Additionally, the p5 promoter may be enhanced by nucleotides 1-173 of SEQ ID NO:1. Furthermore, smaller fragments of p5 promoter that retain promoter activity can readily be determined by standard procedures including, for example, constructing a series of deletions in the p5 promoter, linking the deletion to a reporter gene, and determining whether the reporter gene is expressed, *i.e.*, transcribed and/or translated. The promoter can be the promoter of any of the AAV serotypes, and can be the p19 promoter (SEQ ID NO: 16) or the p40 promoter set forth in the sequence listing as SEQ ID NO: 17.

It should be recognized that any errors in any of the nucleotide sequences disclosed herein can be corrected, for example, by using the hybridization procedure described below with various probes derived from the described sequences such that the coding sequence can be reisolated and resequenced. Rapid screening for point mutations can also be achieved with the use of polymerase chain reaction-single strand

conformation polymorphism (PCR-SSCP). The corresponding amino acid sequence can then be corrected accordingly.

The BAAV-derived vector of the invention can further comprise a heterologous nucleic acid functionally linked to the promoter. By "heterologous nucleic acid" is meant that any heterologous or exogenous nucleic acid, i.e. not normally found in wild-type BAAV can be inserted into the vector for transfer into a cell, tissue or organism. By "functionally linked" is meant that the promoter can promote expression of the heterologous nucleic acid, as is known in the art, and can include the appropriate orientation of the promoter relative to the heterologous nucleic acid. Furthermore, the heterologous nucleic acid preferably has all appropriate sequences for expression of the nucleic acid. The nucleic acid can include, for example, expression control sequences, such as an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

The heterologous nucleic acid can encode beneficial proteins or polypeptides that replace missing or defective proteins required by the cell or subject into which the vector is transferred or can encode a cytotoxic polypeptide that can be directed, *e.g.*, to cancer cells or other cells whose death would be beneficial to the subject. The heterologous nucleic acid can also encode antisense RNAs that can bind to, and thereby inactivate, mRNAs made by the subject that encode harmful proteins. The heterologous nucleic acid can also encode ribozymes that can effect the sequence-specific inhibition of gene expression by the cleavage of mRNAs. In one embodiment, antisense polynucleotides can be produced from a heterologous expression cassette in an AAV5 vector construct where the expression cassette contains a sequence that promotes cell-type specific expression (Wirak *et al.*, *EMBO* 10:289 (1991)). For general methods relating to antisense polynucleotides, see *Antisense RNA and DNA*, D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988).

Examples of heterologous nucleic acids which can be administered to a cell or subject as part of the present BAAV vector can include, but are not limited to the

following: nucleic acids encoding secretory and nonsecretory proteins, nucleic acids encoding therapeutic agents, such as tumor necrosis factors (TNF), such as TNF-α; interferons, such as interferon-α, interferon-β, and interferon-γ; interleukins, such as IL-1, IL-1β, and ILs -2 through -14; GM-CSF; adenosine deaminase; cellular growth factors, such as lymphokines; soluble CD4; Factor VIII; Factor IX; T-cell receptors; LDL receptor; ApoE; ApoC; alpha-1 antitrypsin; ornithine transcarbamylase (OTC); cystic fibrosis transmembrane receptor (CFTR); insulin; Fc receptors for antigen binding domains of antibodies, such as immunoglobulins; anit-HIV decoy tar elements; and antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A, non-B virus. The nucleic acid is chosen considering several factors, including the cell to be transfected. Where the target cell is a blood cell, for example, particularly useful nucleic acids to use are those which allow the blood cells to exert a therapeutic effect, such as a gene encoding a clotting factor for use in treatment of hemophilia. Another target cell is the lung airway cell, which can be used to administer nucleic acids, such as those coding for the cystic fibrosis transmembrane receptor, which could provide a gene therapeutic treatment for cystic fibrosis. Other target cells include muscle cells where useful nucleic acids, such as those encoding cytokines and growth factors, can be transduced and the protein the nucleic acid encodes can be expressed and secreted to exert its effects on other cells, tissues and organs, such as the liver. Furthermore, the nucleic acid can encode more than one gene product, limited only, if the nucleic acid is to be packaged in a capsid, by the size of nucleic acid that can be packaged.

Furthermore, suitable nucleic acids can include those that, when transferred into a primary cell, such as a blood cell, cause the transferred cell to target a site in the body where that cell's presence would be beneficial. For example, blood cells such as TIL cells can be modified, such as by transfer into the cell of a Fab portion of a monoclonal antibody, to recognize a selected antigen. Another example would be to introduce a nucleic acid that would target a therapeutic blood cell to tumor cells. Nucleic acids useful in treating cancer cells include those encoding chemotactic factors which cause an inflammatory response at a specific site, thereby having a therapeutic effect.

Cells, particularly blood cells, muscle cells, airway epithelial cells, brain cells and endothelial cells having such nucleic acids transferred into them can be useful in a variety of diseases, syndromes and conditions. For example, suitable nucleic acids include nucleic acids encoding soluble CD4, used in the treatment of AIDS and α-antitrypsin, used in the treatment of emphysema caused by α-antitrypsin deficiency. Other diseases, syndromes and conditions in which such cells can be useful include, for example, adenosine deaminase deficiency, sickle cell deficiency, brain disorders such as Alzheimer's disease, thalassemia, hemophilia, diabetes, phenylketonuria, growth disorders and heart diseases, such as those caused by alterations in cholesterol metabolism, and defects of the immune system.

Other cells in which a gene of interest can be expressed include, but are not limited to, fibroblasts, neurons, retinal cells, kidney cells, lung cells, bone marrow stem cells, hematopoietic stem cells, retinal cells and neurons. The cells in which the gene of interest can be expressed can be dividing cells such as MDCK cells, BHK cells, HeLa cells, 3T3 cells, CV1 cells, COS7 cells, HOS cells and 293 cells. The cells can also be embryonic stem cells of mouse, rhesus, human, bovine or sheep origin, as well as stem cells of neural, hematopoietic, muscle, cardiac, immune or other origin. Nondividing cells can also be contacted with a particle of the present invention to express a gene of interest. Such cells include, but are not limited to hematopoietic stem cells and embryonic stem cells that have been rendered non-dividing.

As another example, hepatocytes can be transfected with the present vectors having useful nucleic acids to treat liver disease. For example, a nucleic acid encoding OTC can be used to transfect hepatocytes (*ex vivo* and returned to the liver or *in vivo*) to treat congenital hyperammonemia, caused by an inherited deficiency in OTC. Another example is to use a nucleic acid encoding LDL to target hepatocytes *ex vivo* or *in vivo* to treat inherited LDL receptor deficiency. Such transfected hepatocytes can also be used to treat acquired infectious diseases, such as diseases resulting from a viral infection. For example, transduced hepatocyte precursors can be used to treat viral hepatitis, such as hepatitis B and non-A, non-B hepatitis, for example by transducing the hepatocyte precursor with a nucleic acid encoding an antisense RNA that inhibits viral replication. Another example includes transferring a vector of the present

invention having a nucleic acid encoding a protein, such as γ-interferon, which can confer resistance to the hepatitis virus.

For a procedure using transfected hepatocytes or hepatocyte precursors, hepatocyte precursors having a vector of the present invention transferred in can be grown in tissue culture, removed from the tissue culture vessel, and introduced to the body, such as by a surgical method. In this example, the tissue would be placed directly into the liver, or into the body cavity in proximity to the liver, as in a transplant or graft. Alternatively, the cells can simply be directly injected into the liver, into the portal circulatory system, or into the spleen, from which the cells can be transported to the liver via the circulatory system. Furthermore, the cells can be attached to a support, such as microcarrier beads, which can then be introduced, such as by injection, into the peritoneal cavity. Once the cells are in the liver, by whatever means, the cells can then express the nucleic acid and/or differentiate into mature hepatocytes which can express the nucleic acid.

The BAAV-derived vector can include any normally occurring BAAV nucleic acid sequences in addition to an ITR and promoter. The BAAV-derived vector can also include sequences that are at least 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the BAAV nucleic acids set forth herein. Examples of vector constructs are provided below.

The present vector or BAAV particle or recombinant BAAV virion can utilize any unique fragment of these present BAAV nucleic acids, including the BAAV nucleic acids set forth in SEQ ID NOS: 1, 2, 4, 6, 8, 10 and 12-17. To be unique, the fragment must be of sufficient size to distinguish it from other known sequences, most readily determined by comparing any nucleic acid fragment to the nucleotide sequences of nucleic acids in computer databases, such as GenBank. Such comparative searches are standard in the art. Typically, a unique fragment useful as a primer or probe will be at least about 8 or 10, preferable at least 20 or 25 nucleotides in length, depending upon the specific nucleotide content of the sequence. Additionally, fragments can be, for example, at least about 30, 40, 50, 75, 100, 200 or 500 nucleotides in length and can encode polypeptides or be probes. The nucleic acid can be single or double stranded,

depending upon the purpose for which it is intended. Where desired, the nucleic acid can be RNA.

It is understood that as discussed herein the use of the terms "homology" and "identity" mean the same thing as similarity. Thus, for example, if the use of the word homology is used to refer to two non-natural sequences, it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related.

In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed nucleic acids and polypeptides herein, is through defining the variants and derivatives in terms of homology to specific known sequences. In general, variants of nucleic acids and polypeptides herein disclosed typically have at least, about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two polypeptides or nucleic acids. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. MoL Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI; the BLAST algorithm of Tatusova and Madden FEMS Microbiol. Lett. 174: 247-250 (1999) available from the

National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. Science 244:48-52, 1989, Jaeger et al. Proc. Natl. Acad. Sci. USA 86:7706-7710, 1989, Jaeger et al. Methods Enzymol. 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity.

For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

The present invention further provides a BAAV capsid protein to contain the vector. In particular, the present invention provides not only a polypeptide comprising all three BAAV coat proteins, *i.e.*, VP1, VP2 and VP3, but also a polypeptide

comprising each BAAV coat protein individually, SEQ ID NOS: 7, 9, and 11, respectively. Thus, an BAAV particle comprising an BAAV capsid protein comprises at least one BAAV coat protein VP1, VP2 or VP3. A BAAV particle comprising an BAAV capsid protein can be utilized to deliver a nucleic acid vector to a cell, tissue or subject. For example, the herein described BAAV vectors can be encapsidated in an AAV5 capsid-derived particle and utilized in a gene delivery method. Furthermore, other viral nucleic acids can be encapsidated in the BAAV particle and utilized in such delivery methods. For example, an AAV1-8 or AAAV vector (e.g. AAV1-8 or AAAV ITR and nucleic acid of interest)can be encapsidated in an BAAV particle and administered. Furthermore, a BAAV chimeric capsid incorporating both AAV1-8 or AAAV capsid and BAAV capsid sequences can be generated, by standard cloning methods, selecting regions from the known sequences of each protein as desired. For example, particularly antigenic regions of the BAAV capsid protein can be replaced with the corresponding region of the BAAV capsid protein. In addition to chimeric capsids incorporating AAV2 capsid sequences, chimeric capsids incorporating AAV1, 3-8 , and AAV5 capsid sequences can be generated, by standard cloning methods, selecting regions from the known sequences of each protein as desired. Alternatively a chimeric capsid can be made by the addition of a plasmid that expresses AAV1-8 capsid proteins at a ratio with the BAAV capsid expression plasmid that allows only a few capsid proteins to be incorpated into the BAAV particle. Thus, for example, a chimeric particle may be constructed that contains 6 AAV2 capsid proteins and 54 BAAV capsid proteins if the complete capsid contains 60 capsid proteins.

The capsids can also be modified to alter their specific tropism by genetically altering the capsid to encode a specific ligand to a cell surface receptor. Alternatively, the capsid can be chemically modified by conjugating a ligand to a cell surface receptor. By genetically or chemically altering the capsids, the tropism can be modified to direct BAAV to a particular cell or population of cells. The capsids can also be altered immunologically by conjugating the capsid to an antibody that recognizes a specific protein on the target cell or population of cells.

It has been recently reported that insertion of foreign epitopes (RGD motif, LH receptor targeting epitope) in certain regions of AAV2 capsid can redirect viral tropism.

However, AAV2 naturally infects a wide variety of cell types and complete retargeting of rAAV2 would be difficult to achieve. The present invention provides two regions in the capsid of BAAV that are on the virus surface and could tolerate substitution. These two regions are aa 257-264 (GSSNASDT) and aa 444-457 (TTSGGTLNQGNSAT). Other regions of the BAAV capsid could also accommodate the substitution of amino acids that would allow for epitope presentation on the surface of the virus. All of these regions would have in common 1) Surface exposure 2) able to support a substitution of sequence to insert the epitope 3) still allow for capsid assembly.

Because of the symmetry of the AAV particles, a substitution in one subunit of the capsid will appear multiple times on the capsid surface. For example the capsid is made of approximately 55 VP3 proteins. Therefore an epitope incorporated in the VP3 protein could be expressed 55 times on the surface of each particle increasing the likelihood of the epitope forming a stable interaction with its target. In some cases this may be too high of a ligand density for functional binding or this high density of epitope may interfere with capsid formation. The epitope density could be lowered by introducing another plasmid into the packaging system for production of recombinant particles and the ratio between the packaging plasmid with the modified VP3 protein and the wt VP3 protein altered to balance the epitope density on the virus surface.

Epitopes could be incorporated into the virus capsid for the purpose of 1) altering the tropism of the virus 2) blocking an immune response direct at the virus 3) developing a host immune response to the epitope for the purpose of vaccination. Examples of epitopes that could be added to BAAV capsids include but are not limited to:

LH receptor binding epitope

RGD integrin binding epitope

CD13 binding epitope NGRAHA

The Retanef polyprotein vaccine candidate for HIV-1

single chain antibody fragments directed against tumor cells

Endothelial cell binding epitope SIGYPLP

serpin receptor ligand, KFNKPFVFLI

protective B-cell epitope hemagglutinin (HA) 91-108 from influenza HA

NDV B-cell immunodominant epitope (IDE) spanning residues 447 to 455
Major immunogenic epitope for parvovirus B19 (NISLDNPLENPSSLFDLVARIK) that can elicit protective antibody titers.

The capsids can also be assembled into empty particles by expression in mammalian, bacterial, fungal or insect cells. For example, AAV2 particles are known to be made from VP3 and VP2 capsid proteins in baculovirus. The same basic protocol can produce an empty BAAV particle comprising BAAV capsid proteins and also full particles.

The herein described recombinant BAAV nucleic acid derived vector can be encapsidated in an AAV particle. In particular, it can be encapsidated in an AAV1 particle, an AAV2 particle, an AAV3 particle, an AAV4 particle, an AAV5 particle or an AAV6 or AAV7 or an AAV8 or AAAV particle, a portion of any of these capsids, or a chimeric capsid particle as described above, by standard methods using the appropriate capsid proteins in the encapsidation process, as long as the nucleic acid vector fits within the size limitation of the particle utilized. The encapsidation process itself is standard in the art. The BAAV replication machinery, i.e. the rep initiator proteins and other functions required for replication, can be utilized to produce the BAAV genome that can be packaged in an AAV1-8 or AAAV capsid.

The recombinant BAAV virion containing a vector can also be produced by recombinant methods utilizing multiple plasmids. In one example, the BAAV rep nucleic acid would be cloned into one plasmid, the BAAV ITR nucleic acid would be cloned into another plasmid and the AAV1-8 capsid nucleic acid would be cloned on another plasmid. These plasmids would then be introduced into cells. The cells that were efficiently transduced by all three plasmids, would exhibit specific integration as well as the ability to produce BAAV recombinant virus. Additionally, two plasmids could be used where the BAAV rep nucleic acid would be cloned into one plasmid and the BAAV ITR and BAAV capsid would be cloned into another plasmid. These plasmids would then be introduced into cells. The cells that were efficiently transduced by both plasmids, would exhibit specific integration as well as the ability to produce BAAV recombinant virus.

An BAAV capsid polypeptide encoding the entire VP1, VP2, and VP3 polypeptide can overall have greater than 56% homology to the polypeptide having the amino acid sequence encoded by nucleotides in SEQ ID NOS:6, 8 and 10. The capsid protein can have about 70% homology, about 75% homology, 80% homology, 85% homology, 90% homology, 95% homology, 98% homology, 99% homology, or even 100% homology to the protein having the amino acid sequence encoded by the nucleotides set forth in SEQ ID NOS:6, 8 or 10. The percent homology used to identify proteins herein, can be based on a nucleotide-by-nucleotide comparison or more preferable is based on a computerized algorithm as described herein. Variations in the amino acid sequence of the BAAV capsid protein are contemplated herein, as long as the resulting particle comprising an BAAV capsid protein remains antigenically or immunologically distinct from AAV1-8 or AAAV capsid, as can be routinely determined by standard methods. Specifically, for example, ELISA and Western blots can be used to determine whether a viral particle is antigenically or immunologically distinct from AAV2 or the other serotypes. Furthermore, the BAAV particle preferably retains tissue tropism distinction from other AAVs, such as that exemplified in the examples herein. A BAAV chimeric particle comprising at least one BAAV coat protein may have a different tissue tropism from that of an BAAV particle consisting only of BAAV coat proteins, but is still distinct from the tropism of an AAV2 particle.

The invention further provides a recombinant BAAV virion, comprising a BAAV particle containing, *i.e.*, encapsidating, a vector comprising a pair of BAAV inverted terminal repeats. The recombinant vector can further comprise a BAAV Rep-encoding nucleic acid. The vector encapsidated in the particle can further comprise an exogenous nucleic acid inserted between the inverted terminal repeats.

The invention further contemplates chimeric recombinant ITRs that contain a rep binding site and a TRS site recognized by that Rep protein. By "Rep protein" is meant all four of the Rep proteins, Rep 40, Rep 78, Rep 52, Rep 68. Alternatively, "Rep protein" could be one or more of the Rep proteins described herein. One example of a chimeric ITR would consist of an BAAV D region (SEQ ID NO: 13), an BAAV

TRS site (SEQ ID NO: 14), an AAV2 hairpin and an AAV2 Rep binding site. Another example would be a BAAV D region, an BAAV TRS site, an AAV3 hairpin and an AAV3 Rep binding site. In these chimeric ITRs, the D region can be from AAV1-8 or AAAV. The hairpin can be derived from AAV 1-8 or AAAV. The binding site can be derived from any of AAV1-8 or AAAV. Preferably, the D region and the TRS are from the same serotype.

The chimeric ITRs can be combined with BAAV Rep protein and any of the AAV serotype capsids to obtain recombinant virion. For example, recombinant virion can be produced by a BAAV D region, an BAAV TRS site, an AAV2 hairpin, an AAV2 binding site, BAAV Rep protein and AAV1 capsid. This recombinant virion would possess the cellular tropism conferred by the AAV1 capsid protein and would possess the efficient replication conferred by the BAAV Rep.

Other examples of the ITR, Rep protein and Capsids that will produce recombinant virus are provided in the list below but not limited to :

BAAV ITR + BAAV Rep + BAAV Cap=virus
AAV5 ITR + BAAV Rep + BAAV Cap=virus
AAV5 ITR + BAAV Rep + AAV1 Cap=virus
AAV5 ITR + BAAV Rep + AAV2 Cap=virus
AAV5 ITR + BAAV Rep + AAV3 Cap=virus
AAV5 ITR + BAAV Rep + AAV4 Cap=virus
AAV5 ITR + BAAV Rep + AAV5 Cap=virus
AAV5 ITR + BAAV Rep + AAV6 Cap=virus
AAV5 ITR + BAAV Rep + AAV7 Cap=virus
AAV5 ITR + BAAV Rep + AAV8 Cap=virus
BAAV ITR + AAV5 Rep + BAAV Cap=virus
BAAV ITR + AAV5 Rep + AAV1 Cap=virus
BAAV ITR + AAV5 Rep + AAV2 Cap=virus
BAAV ITR + AAV5 Rep + AAV3 Cap=virus
BAAV ITR + AAV5 Rep + AAV4 Cap=virus
BAAV ITR + AAV5 Rep + AAV5 Cap=virus
BAAV ITR + AAV5 Rep + AAV6 Cap=virus

BAAV ITR + AAV5 Rep + AAV7 Cap=virus
BAAV ITR + AAV5 Rep + AAV8 Cap=virus
AAV5 ITR + AAV5 Rep + BAAV Cap=virus
AAV1 ITR + AAV1 Rep + BAAV Cap=virus
AAV2 ITR + AAV2 Rep + BAAV Cap=virus
AAV3 ITR + AAV3 Rep + BAAV Cap=virus
AAV4 ITR + AAV4 Rep + BAAV Cap=virus
AAV5 ITR + AAV5 Rep + BAAV Cap=virus
AAV6 ITR + AAV6 Rep + BAAV Cap=virus
AAV7 ITR + AAV7 Rep + BAAV Cap=virus
AAV8 ITR + AAV8 Rep + BAAV Cap=virus

One of skill in the art would know how to employ standard techniques to obtain the sequences from any of AAV 1-8 in order to combine them with BAAV sequences. Examples of BAAV sequences that can be utilized in these constructs can be found under Accession No. AY388617 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV1 sequences that can be utilized in these constructs can be found in GenBank under Accession No. AF063497 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV2 sequences that can be utilized in these constructs can be found in GenBank under Accession No. AF043303 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV3 sequences that can be utilized in these constructs can be found in GenBank under Accession No. NC_001729 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV4 sequences that can be utilized in these constructs can be found in GenBank under Accession No. U89790 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV5 sequences that can be utilized in these constructs can be found in GenBank under Accession No. AF085716 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV6 sequences that can be utilized in these constructs can be found in GenBank under Accession No. NC_001862 and AF028704 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV7 sequences that

can be utilized in these constructs can be found in GenBank under Accession No. AF513851 and these sequences are hereby incorporated in their entireties by this reference. Examples of AAV8 sequences that can be utilized in these constructs can be found in GenBank under Accession No. AF513852 and these sequences are hereby incorporated in their entireties by this reference. In any of the constructs described herein, inclusion of a promoter is preferred. As used in the constructs herein, unless otherwise specified, Cap (capsid) refers to any of BAAV VP1, BAAV VP2, BAAV VP3, combinations thereof, functional fragments of any of VP1, VP2 or VP3, or chimeric capsids as described herein. The ITRs of the constructs described herein, can be chimeric recombinant ITRs as described elsewhere in the application.

Conjugates of recombinant or wild-type BAAV virions and nucleic acids or proteins can be used to deliver those molecules to a cell. For example, the purified BAAV can be used as a vehicle for delivering DNA bound to the exterior of the virus. Examples of this are to conjugate the DNA to the virion by a bridge using poly-L-lysine or other charged molecule. Also contemplated are virosomes that contain BAAV structural proteins (BAAV capsid proteins), lipids such as DOTAP, and nucleic acids that are complexed via charge interaction to introduce DNA into cells.

Also provided by this invention are conjugates that utilize the BAAV capsid or a unique region of the BAAV capsid protein (e.g. VP1, VP2 or VP3 or combinations thereof) to introduce DNA into cells. For example, the BAAV VP3 protein or fragment thereof, can be conjugated to a DNA on a plasmid that is conjugated to a lipid. Cells can be infected using the targeting ability of the VP3 capsid protein to achieve the desired tissue tropism, specific to BAAV. BAAV VP1 and VP2 proteins can also be utilized to introduce DNA or other molecules into cells. By further incorporating the Rep protein and the AAV TRS into the DNA-containing conjugate, cells can be transduced and targeted integration can be achieved. For example, if BAAV specific targeted integration is desired, a conjugate composed of the BAAV VP3 capsid, BAAV rep or a fragment of BAAV rep, BAAV TRS, the rep binding site, the heterologous DNA of interest, and a lipid, can be utilized to achieve BAAV specific tropism and BAAV specific targeted integration in the genome.

Further provided by this invention are chimeric viruses where BAAV can be combined with herpes virus, baculovirus or other viruses to achieve a desired tropism associated with another virus. For example, the BAAV ITRs could be inserted in the herpes virus and cells could be infected. Post-infection, the ITRs of BAAV could be acted on by BAAV rep provided in the system or in a separate vehicle to rescue BAAV from the genome. Therefore, the cellular tropism of the herpes simplex virus can be combined with BAAV rep mediated targeted integration. Other viruses that could be utilized to construct chimeric viruses include, lentivirus, retrovirus, pseudotyped retroviral vectors, and adenoviral vectors.

The present invention further provides isolated nucleic acids of BAAV. For example, provided is an isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:1 (BAAV genome). This nucleic acid, or portions thereof, can be inserted into vectors, such as plasmids, yeast artificial chromosomes, or other viral vector (particle), if desired, by standard cloning methods. The present invention also provides an isolated nucleic acid consisting essentially of the nucleotide sequence set. forth in SEQ ID NO:1. The nucleotides of SEQ ID NO:1 can have minor modifications and still be contemplated by the present invention. For example, modifications that do not alter the amino acid encoded by any given codon (such as by modification of the third, "wobble," position in a codon) can readily be made, and such alterations are known in the art. Furthermore, modifications that cause a resulting neutral (conserved) amino acid substitution of a similar amino acid can be made in a coding region of the genome. Additionally, modifications as described herein for the BAAV components, such as the ITRs, the p5 promoter, etc. are contemplated in this invention. Furthermore, modifications to regions of SEQ ID NO:1 other than in the ITR, TRS, Rep binding site and hairpin are likely to be tolerated without serious impact on the function of the nucleic acid as a recombinant vector.

As used herein, the term "isolated" refers to a nucleic acid separated or significantly free from at least some of the other components of the naturally occurring organism, for example, the cell structural components or viral components commonly found associated with nucleic acids in the environment of the virus and/or other nucleic acids. The isolation of the native nucleic acids can be accomplished, for example, by

techniques such as cell lysis followed by phenol plus chloroform extraction, followed by ethanol precipitation of the nucleic acids. The nucleic acids of this invention can be isolated from cells according to any of many methods well known in the art.

As used herein, the term "nucleic acid" refers to single-or multiple stranded molecules which may be DNA or RNA, or any combination thereof, including modifications to those nucleic acids. The nucleic acid may represent a coding strand or its complement, or any combination thereof. Nucleic acids may be identical in sequence to the sequences which are naturally occurring for any of the novel genes discussed herein or may include alternative codons which encode the same amino acid as those provided herein, including that which is found in the naturally occurring sequence. These nucleic acids can also be modified from their typical structure. Such modifications include, but are not limited to, methylated nucleic acids, the substitution of a non-bridging oxygen on the phosphate residue with either a sulfur (yielding phosphorothioate deoxynucleotides), selenium (yielding phosphorselenoate deoxynucleotides), or methyl groups (yielding methylphosphonate deoxynucleotides).

The present invention additionally provides an isolated nucleic acid that selectively hybridizes with any nucleic acid disclosed herein, including the entire BAAV genome and any unique fragment thereof, including the Rep and capsid encoding sequences (e.g. SEQ ID NOS: 1, 2, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17). Specifically, the nucleic acid can selectively or specifically hybridize to an isolated nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO:1 (BAAV genome). The present invention further provides an isolated nucleic acid that selectively or specifically hybridizes with an isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:1 (BAAV genome). By "selectively hybridizes" as used herein is meant a nucleic acid that hybridizes to one of the disclosed nucleic acids under sufficient stringency conditions without significant hybridization to a nucleic acid encoding an unrelated protein, and particularly, without detectably hybridizing to nucleic acids of AAV2. Thus, a nucleic acid that selectively hybridizes with a nucleic acid of the present invention will not selectively hybridize under stringent conditions with a nucleic acid encoding a different protein or the

corresponding protein from a different serotype of the virus, and vice versa. A "specifically hybridizing" nucleic acid is one that hybridizes under stringent conditions to only a nucleic acid found in BAAV. Therefore, nucleic acids for use, for example, as primers and probes to detect or amplify the target nucleic acids are contemplated herein. Nucleic acid fragments that selectively hybridize to any given nucleic acid can be used, *e.g.*, as primers and or probes for further hybridization or for amplification methods (*e.g.*, polymerase chain reaction (PCR), ligase chain reaction (LCR)). Additionally, for example, a primer or probe can be designed that selectively hybridizes with both BAAV and a gene of interest carried within the BAAV vector (*i.e.*, a chimeric nucleic acid).

Stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. Typically, the stringency of hybridization to achieve selective hybridization involves hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the T_m (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the T_m. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The washing temperatures can be used as described above to achieve selective stringency, as is known in the art. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. *Methods Enzymol.* 1987:154:367, 1987). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched

for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

A nucleic acid that selectively hybridizes to any portion of the BAAV genome is contemplated herein. Therefore, a nucleic acid that selectively hybridizes to BAAV can be of longer length than the BAAV genome, it can be about the same length as the BAAV genome or it can be shorter than the BAAV genome. The length of the nucleic acid is limited on the shorter end of the size range only by its specificity for hybridization to BAAV, *i.e.*, once it is too short, typically less than about 5 to 7 nucleotides in length, it will no longer bind specifically to BAAV, but rather will hybridize to numerous background nucleic acids. Additionally contemplated by this invention is a nucleic acid that has a portion that specifically hybridizes to BAAV and a portion that specifically hybridizes to a gene of interest inserted within BAAV.

The present invention further provides an isolated nucleic acid encoding a bovine adeno-associated virus Rep protein. The BAAV Rep proteins are encoded by open reading frame (ORF) 1 of the BAAV genome. Examples of the BAAV Rep genes are shown in the nucleic acid set forth in SEQ ID NO:1, and include nucleic acids consisting essentially of the nucleotide sequences set forth in SEQ ID NOS:2 (rep78), 4(rep52) and nucleic acids comprising the nucleotide sequences set forth in SEQ ID NOS:2 and 4. However, the present invention contemplates that the Rep nucleic acid can include any one, two, three, or four of the four Rep proteins, in any order, in such a nucleic acid. Furthermore, minor modifications are contemplated in the nucleic acid, such as silent mutations in the coding sequences, mutations that make neutral or conservative changes in the encoded amino acid sequence, and mutations in regulatory regions that do not disrupt the expression of the gene. Examples of other minor modifications are known in the art. Further modifications can be made in the nucleic acid, such as to disrupt or alter expression of one or more of the Rep proteins in order to, for example, determine the effect of such a disruption; such as to mutate one or more of the Rep proteins to determine the resulting effect, etc. However, in general, a modified nucleic acid encoding a Rep protein will have at least about 85%, about 90%,

about 93%, about 95%, about 98% or 100% homology to the Rep nucleic sequences described herein e.g., SEQ ID NOS: 2, and 4, and the Rep polypeptide encoded therein will have overall about 93%, about 95%, about 98%, about 99% or 100% homology with the amino acid sequence described herein, e.g., SEQ ID NOS:3 and 5. Percent homology is determined by the techniques described herein.

The present invention also provides an isolated nucleic acid that selectively or specifically hybridizes with a nucleic acid consisting essentially of the nucleotide sequence set forth in SEQ ID NOS:2 and 4, and an isolated nucleic acid that selectively hybridizes with a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NOS:2 and 4. "Selectively hybridizing" and "stringency of hybridization" is defined elsewhere herein.

As described above, the present invention provides the nucleic acid encoding a Rep 78 protein and, in particular an isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO: 2, an isolated nucleic acid consisting essentially of the nucleotide sequence set forth in SEQ ID NO: 2, and a nucleic acid encoding the bovine adeno-associated virus protein having the amino acid sequence set forth in SEQ ID NO: 3. The present invention also provides the nucleic acid encoding a Rep 52 protein, and in particular an isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:4, an isolated nucleic acid consisting essentially of the nucleotide sequence set forth in SEQ ID NO:4, and a nucleic acid encoding the bovine adeno-associated virus Rep 52 protein having the amino acid sequence set forth in SEQ ID NO:5. As described elsewhere herein, these nucleic acids can have minor modifications, including silent nucleotide substitutions, mutations causing conservative amino acid substitutions in the encoded proteins, and mutations in control regions that do not or minimally affect the encoded amino acid sequence.

The present invention further provides a nucleic acid encoding the entire BAAV Capsid polypeptide. Furthermore, the present invention provides a nucleic acid encoding each of the three BAAV coat proteins, VP1, VP2, and VP3. Thus, the present invention provides a nucleic acid encoding BAAV VP1, a nucleic acid encoding

BAAV VP2, and a nucleic acid encoding BAAV VP3. Thus, the present invention provides a nucleic acid encoding the amino acid sequence set forth in SEQ ID NO:7 (VP1); a nucleic acid encoding the amino acid sequence set forth in SEQ ID NO:9 (VP2), and a nucleic acid encoding the amino acid sequence set forth in SEQ ID NO:11 (VP3). The present invention also specifically provides a nucleic acid comprising SEQ ID NO:6 (VP1 gene); a nucleic acid comprising SEQ ID NO:8 (VP2 gene); and a nucleic acid comprising SEQ ID NO:10 (VP3 gene). The present invention also specifically provides a nucleic acid consisting essentially of SEQ ID NO:6 (VP1 gene), a nucleic acid consisting essentially of SEQ ID NO:8 (VP2 gene), and a nucleic acid consisting essentially of SEQ ID NO:10 (VP3 gene). Minor modifications in the nucleotide sequences encoding the capsid, or coat, proteins are contemplated, as described above for other BAAV nucleic acids. However, in general, a modified nucleic acid encoding a capsid protein will have at least about 85%, about 90%, about 93%, about 95%, about 98% or 100% homology to the capsid nucleic sequences described herein e.g., SEQ ID NOS: 6, 8, and 10, and the capsid polypeptide encoded therein will have overall about 93%, about 95%, about 98%, about 99% or 100% homology with the amino acid sequence described herein, e.g., SEQ ID NOS:7, 9, and 11. Nucleic acids that selectively hybridize with the nucleic acids of SEQ ID NOS:6, 8 and 10 under the conditions described above are also provided.

The present invention also provides a cell containing one or more of the herein described nucleic acids, such as the BAAV genome, BAAV ORF1 and ORF2, each BAAV Rep protein gene, or each BAAV capsid protein gene. Such a cell can be any desired cell and can be selected based upon the use intended. For example, cells can include bacterial cells, yeast cells, insect cells, human HeLa cells and simian Cos cells as well as other human and mammalian cells and cell lines. Primary cultures as well as established cultures and cell lines can be used. Nucleic acids of the present invention can be delivered into cells by any ~~selected~~ means, in particular depending upon the target cells. Many delivery means are well-known in the art. For example, electroporation, calcium phosphate precipitation, microinjection, cationic or anionic liposomes, and liposomes in combination with a nuclear localization signal peptide for delivery to the nucleus can be utilized, as is known in the art. Additionally, if the

nucleic acids are in a viral particle, the cells can simply be transduced with the virion by standard means known in the art for AAV transduction. Small amounts of the recombinant BAAV virus can be made to infect cells and produce more of itself.

The invention provides purified BAAV polypeptides. The term "polypeptide" as used herein refers to a polymer of amino acids and includes full-length proteins and fragments thereof. Thus, "protein," polypeptide," and "peptide" are often used interchangeably herein. Substitutions can be selected by known parameters to be neutral (*see, e.g.,* Robinson WE Jr, and Mitchell WM., AIDS 4:S151-S162 (1990)). As will be appreciated by those skilled in the art, the invention also includes those polypeptides having slight variations in amino acid sequences or other properties. Such variations may arise naturally as allelic variations (*e.g.,* due to genetic polymorphism) or may be produced by human intervention (*e.g.,* by mutagenesis of cloned DNA sequences), such as induced point, deletion, insertion and substitution mutants. Minor changes in amino acid sequence are generally preferred, such as conservative amino acid replacements, small internal deletions or insertions, and additions or deletions at the ends of the molecules. Substitutions may be designed based on, for example, the model of Dayhoff, *et al.* (in *Atlas of Protein Sequence and Structure 1978*, Nat'l Biomed. Res. Found., Washington, D.C.). These modifications can result in changes in the amino acid sequence, provide silent mutations, modify a restriction site, or provide other specific mutations. The location of any modifications to the polypeptide will often determine its impact on function. Particularly, alterations in regions non-essential to protein function will be tolerated with fewer effects on function. Elsewhere in the application regions of the BAAV proteins are described to provide guidance as to where substitutions, additions or deletions can be made to minimize the likelihood of disturbing the function of the variant.

A polypeptide of the present invention can be readily obtained by any of several means. For example, the polypeptide of interest can be synthesized chemically by standard methods. Additionally, the coding regions of the genes can be recombinantly expressed and the resulting polypeptide isolated by standard methods. Furthermore, an

antibody specific for the resulting polypeptide can be raised by standard methods (see, *e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988), and the protein can be isolated from a cell expressing the nucleic acid encoding the polypeptide by selective hybridization with the antibody. This protein can be purified to the extent desired by standard methods of protein purification (see, *e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

Typically, to be unique, a polypeptide fragment of the present invention will be at least about 5 amino acids in length; however, unique fragments can be 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acids in length. A unique polypeptide will typically comprise such a unique fragment; however, a unique polypeptide can also be determined by its overall homology. A unique polypeptide can be 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acids in length. Uniqueness of a polypeptide fragment can readily be determined by standard methods such as searches of computer databases of known peptide or nucleic acid sequences or by hybridization studies to the nucleic acid encoding the protein or to the protein itself, as known in the art. The uniqueness of a polypeptide fragment can also be determined immunologically as well as functionally. Uniqueness can be simply determined in an amino acid-by-amino acid comparison of the polypeptides.

An antigenic or immunoreactive fragment of this invention is typically an amino acid sequence of at least about 5 consecutive amino acids, and it can be derived from the BAAV polypeptide amino acid sequence. An antigenic BAAV fragment is any fragment unique to the BAAV protein, as described herein, against which an BAAV-specific antibody can be raised, by standard methods. Thus, the resulting antibody-antigen reaction should be specific for BAAV.

The present invention provides an isolated BAAV Rep protein. An BAAV Rep polypeptide is encoded by ORF1 of BAAV. The present invention also provides each individual BAAV Rep protein. Thus the present invention provides BAAV Rep 52 (e.g., SEQ ID NO: 4), or a unique fragment thereof. The present invention provides BAAV Rep 78 (e.g., SEQ ID NO: 2), or a unique fragment thereof. By "unique

fragment thereof" is meant any smaller polypeptide fragment encoded by an BAAV rep gene that is of sufficient length to be found only in the Rep polypeptide. Substitutions and modifications of the amino acid sequence can be made as described above and, further, can include protein processing modifications, such as glycosylation, to the polypeptide.

The present invention further provides a BAAV Capsid polypeptide or a unique fragment thereof. BAAV capsid polypeptide is encoded by ORF 2 of BAAV. The present invention further provides the individual BAAV capsid proteins, VP1, VP2 and VP3 or unique fragments thereof. Thus, the present invention provides an isolated polypeptide having the amino acid sequence set forth in SEQ ID NO:6 (VP1). The present invention additionally provides an isolated polypeptide having the amino acid sequence set forth in SEQ ID NO:8 (VP2). The present invention also provides an isolated polypeptide having the amino acid sequence set forth in SEQ ID NO:10 (VP3). By "unique fragment thereof" is meant any smaller polypeptide fragment encoded by any BAAV capsid gene that is of sufficient length to be found only in the BAAV capsid protein. Substitutions and modifications of the amino acid sequence can be made as described above and, further, can include protein processing modifications, such as glycosylation, to the polypeptide. However, an BAAV Capsid polypeptide including all three coat proteins will have greater than about 56% overall homology to the polypeptide encoded by the nucleotides set forth in SEQ ID NOS:6, 8 or 10. The protein can have about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, 93%, 95%, 97% or even 100% homology to the amino acid sequence encoded by the nucleotides set forth in SEQ ID NOS:6, 8 or 10. An BAAV VP1 polypeptide can have at least about 58%, about 60%, about 70%, about 80%, about 90%, 93%, 95%, 97% or about 100% homology to the amino acid sequence set forth in SEQ ID NO:7. An BAAV VP2 polypeptide can have at least about 58%, about 60%, about 70%, about 80%, about 90%, 93%, 95%, 97% or about 100% homology to the amino acid sequence set forth in SEQ ID NO:9. An BAAV VP3 polypeptide can have at least about 60%, about 70%, about 80%, about 90%, 93%, 95%, 97% or about 100% homology to the amino acid sequence set forth in SEQ ID NO:11.

The present invention further provides an isolated antibody that specifically binds a BAAV Rep protein or a unique epitope thereof. Also provided are isolated antibodies that specifically bind the BAAV Rep 52 protein and the BAAV Rep 78 protein having the amino acid sequences set forth in SEQ ID NO: 5 and SEQ ID NO: 3, respectively or that specifically binds a unique fragment thereof. Clearly, any given antibody can recognize and bind one of a number of possible epitopes present in the polypeptide; thus only a unique portion of a polypeptide (having the epitope) may need to be present in an assay to determine if the antibody specifically binds the polypeptide.

The present invention additionally provides an isolated antibody that specifically binds any of the bovine adeno-associated virus capsid proteins (VP1, VP2 or VP3), a unique epitope thereof, or the polypeptide comprising all three BAAV coat proteins. Also provided is an isolated antibody that specifically binds the BAAV capsid protein having the amino acid sequence set forth in SEQ ID NO:7 (VP1), or that specifically binds a unique fragment thereof. The present invention further provides an isolated antibody that specifically binds the BAAV Capsid protein having the amino acid sequence set forth in SEQ ID NO:9 (VP2), or that specifically binds a unique fragment thereof. The invention additionally provides an isolated antibody that specifically binds the BAAV Capsid protein having the amino acid sequence set forth in SEQ ID NO:11 (VP3), or that specifically binds a unique fragment thereof. Again, any given antibody can recognize and bind one of a number of possible epitopes present in the polypeptide; thus only a unique portion of a polypeptide (having the epitope) may need to be present in an assay to determine if the antibody specifically binds the polypeptide.

The antibody can be a component of a composition that comprises an antibody that specifically binds the BAAV protein. The composition can further comprise, *e.g.*, serum, serum-free medium, or a pharmaceutically acceptable carrier such as physiological saline, etc.

By "an antibody that specifically binds" an BAAV polypeptide or protein is meant an antibody that selectively binds to an epitope on any portion of the BAAV peptide such that the antibody binds specifically to the corresponding BAAV polypeptide without significant background. Specific binding by an antibody further

means that the antibody can be used to selectively remove the target polypeptide from a sample comprising the polypeptide or and can readily be determined by radioimmunoassay (RIA), bioassay, or enzyme-linked immunosorbant (ELISA) technology. An ELISA method effective for the detection of the specific antibody-antigen binding can, for example, be as follows: (1) bind the antibody to a substrate; (2) contact the bound antibody with a sample containing the antigen; (3) contact the above with a secondary antibody bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a color reagent; (6) observe the color change.

An antibody can include antibody fragments such as Fab fragments which retain the binding activity. Antibodies can be made as described in, *e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988). Briefly, purified antigen can be injected into an animal in an amount and in intervals sufficient to elicit an immune response. Antibodies can either be purified directly, or spleen cells can be obtained from the animal. The cells are then fused with an immortal cell line and screened for antibody secretion. Individual hybridomas are then propagated as individual clones serving as a source for a particular monoclonal antibody.

The present invention additionally provides a method of screening a cell for infectivity by BAAV comprising contacting the cell with BAAV and detecting the presence of BAAV in the cells. BAAV particles can be detected using any standard physical or biochemical methods. For example, physical methods that can be used for this detection include DNA based methods such as 1) polymerase chain reaction (PCR) for viral DNA or RNA or 2) direct hybridization with labeled probes, and immunological methods such as by 3) antibody directed against the viral structural or non- structural proteins. Catalytic methods of viral detection include, but are not limited to, detection of site and strand specific DNA nicking activity of Rep proteins or replication of an AAV origin- containing substrate. Reporter genes can also be utilized to detect cells that transduct BAAV. For example, β-gal, green flourescent protein or luciferase can be inserted into a recombinant BAAV. The cell can then be contacted

with the recombinant BAAV, either *in vitro* or *in vivo* and a colorimetric assay could detect a color change in the cells that would indicate transduction of BAAV in the cell. Additional detection methods are outlined in Fields, *Virology*, Raven Press, New York, New York. 1996.

For screening a cell for infectivity by BAAV, wherein the presence of BAAV in the cells is determined by nucleic acid hybridization methods, a nucleic acid probe for such detection can comprise, for example, a unique fragment of any of the BAAV nucleic acids provided herein. The uniqueness of any nucleic acid probe can readily be determined as described herein. Additionally, the presence of BAAV in cells can be determined by flourescence, antibodies to gene products, focus forming assays, plaque lifts, Western blots and chromogenic assays. The nucleic acid can be, for example, the nucleic acid whose nucleotide sequence is set forth in SEQ ID NO: 1,3, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17 or a unique fragment thereof.

The present invention includes a method of determining the suitability of an BAAV vector for administration to a subject comprising administering to an antibody-containing sample from the subject an antigenic fragment of an isolated BAAV Rep or Capsid protein, and detecting neutralizing antibody-antigen reaction in the sample, the presence of a neutralizing reaction indicating the BAAV vector may be unsuitable for use in the subject. The present method of determining the suitability of an BAAV vector for administration to a subject can comprise contacting an antibody-containing sample from the subject with a unique antigenic or immunogenic fragment of an BAAV Rep protein (e.g. Rep 52, Rep 78) and detecting an antibody-antigen reaction in the sample, the presence of a reaction indicating the BAAV vector to be unsuitable for use in the subject. The BAAV Rep proteins are provided herein, and their antigenic fragments are routinely determined. The BAAV capsid protein can be used to select an antigenic or immunogenic fragment, for example from the amino acid sequence set forth in SEQ ID NO:7 (VP1), the amino acid sequence set forth in SEQ ID NO: 9 (VP2) or the amino acid sequence set forth in SEQ ID NO:11 (VP3). Alternatively, or additionally, an antigenic or immunogenic fragment of an isolated BAAV Rep protein can be utilized in this determination method. The BAAV Rep protein from which an

antigenic fragment is selected can have the amino acid sequence encoded by the nucleic acid set forth in SEQ ID NO:1, the amino acid sequence set forth in SEQ ID NO:2, or the amino acid sequence set forth in SEQ ID NO:4, the amino acid sequence set forth in SEQ ID NO: 3, or the amino acid sequence set forth in SEQ ID NO:5.

The BAAV polypeptide fragments can be analyzed to determine their antigenicity, immunogenicity and/or specificity. Briefly, various concentrations of a putative immunogenically specific fragment are prepared and administered to a subject and the immunological response (e.g., the production of antibodies or cell mediated immunity) of an animal to each concentration is determined. The amounts of antigen administered depend on the subject, e.g. a human, rabbit or a guinea pig, the condition of the subject, the size of the subject, etc. Thereafter an animal so inoculated with the antigen can be exposed to the BAAV viral particle or BAAV protein to test the immunoreactivity or the antigenicity of the specific immunogenic fragment. The specificity of a putative antigenic or immunogenic fragment can be ascertained by testing sera, other fluids or lymphocytes from the inoculated animal for cross reactivity with other closely related viruses, such as AAV1-8 or AAAV.

By the "suitability of an BAAV vector for administration to a subject" is meant a determination of whether the BAAV vector will elicit a neutralizing immune response upon administration to a particular subject. A vector that does not elicit a significant immune response is a potentially suitable vector, whereas a vector that elicits a significant, neutralizing immune response (e.g. at least 90%) is thus likely to be unsuitable for use in that subject. Significance of any detectable immune response is a standard parameter understood by the skilled artisan in the field. For example, one can incubate the subject's serum with the virus, then determine whether that virus retains its ability to transduce cells in culture. If such virus cannot transduce cells in culture, the vector likely has elicited a significant immune response.

Alternatively, or additionally, one skilled in the art could determine whether or not BAAV administration would be suitable for a particular cell type of a subject. For example, the artisan could culture muscle cells *in vitro* and transduce the cells with BAAV in the presence or absence of the subject's serum. If there is a reduction in

transduction efficiency, this could indicate the presence of a neutralizing antibody or other factors that may inhibit transduction. Normally, greater than 90% inhibition would have to be observed in order to rule out the use of BAAV as a vector. However, this limitation could be overcome by treating the subject with an immunosuppressant that could block the factors inhibiting transduction.

As will be recognized by those skilled in the art, numerous types of immunoassays are available for use in the present invention to detect binding between an antibody and a BAAV polypeptide of this invention. For instance, direct and indirect binding assays, competitive assays, sandwich assays, and the like, as are generally described in, *e.g.*, U.S. Pat. Nos. 4,642,285; 4,376,110; 4,016,043; 3,879,262; 3,852,157; 3,850,752; 3,839,153; 3,791,932; and Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, N.Y. (1988). For example, enzyme immunoassays such as immunofluorescence assays (IFA), enzyme linked immunosorbent assays (ELISA) and immunoblotting can be readily adapted to accomplish the detection of the antibody. An ELISA method effective for the detection of the antibody bound to the antigen can, for example, be as follows: (1) bind the antigen to a substrate; (2) contact the bound antigen with a fluid or tissue sample containing the antibody; (3) contact the above with a secondary antibody specific for the antigen and bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a color reagent; (6) observe color change.

The antibody-containing sample of this method can comprise any biological sample which would contain the antibody or a cell containing the antibody, such as blood, plasma, serum, bone marrow, saliva and urine.

The present invention also provides a method of producing the BAAV virus by transducing a cell with the nucleic acid encoding the virus.

The present method further provides a method of delivering an exogenous (heterologous) nucleic acid to a cell comprising administering to the cell an BAAV particle containing a vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to the cell.

The AAV ITRs in the vector for the herein described delivery methods can be AAV ITRs (SEQ ID NOS: 12). Furthermore, the AAV ITRs in the vector for the herein described nucleic acid delivery methods can also comprise AAV1-8 or AAAV inverted terminal repeats.

The present invention also includes a method of delivering a heterologous nucleic acid to a subject comprising administering to a cell from the subject an BAAV particle containing a vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, and returning the cell to the subject, thereby delivering the nucleic acid to the subject. The AAV ITRs can be any AAV ITRs, including BAAV ITRs, AAV5 ITRs and AAV2 ITRs. For example, in an *ex vivo* administration, cells are isolated from a subject by standard means according to the cell type and placed in appropriate culture medium, again according to cell type (*see, e.g.*, ATCC catalog).. Viral particles are then contacted with the cells as described above, and the virus is allowed to transduce the cells. Cells can then be transplanted back into the subject's body, again by means standard for the cell type and tissue (*e. g.*, in general, U.S. Patent No. 5,399,346; for neural cells, Dunnett, S.B. and Björklund, A., eds., *Transplantation: Neural Transplantation-A Practical Approach,* Oxford University Press, Oxford (1992)). If desired, prior to transplantation, the cells can be studied for degree of transduction by the virus, by known detection means and as described herein. Cells for *ex vivo* transduction followed by transplantation into a subject can be selected from those listed above, or can be any other selected cell. Preferably, a selected cell type is examined for its capability to be transfected by BAAV. Preferably, the selected cell will be a cell readily transduced with BAAV particles; however, depending upon the application, even cells with relatively low transduction efficiencies can be useful, particularly if the cell is from a tissue or organ in which even production of a small amount of the protein or antisense RNA encoded by the vector will be beneficial to the subject.

The present invention further provides a method of delivering a nucleic acid to a cell in a subject comprising administering to the subject an BAAV particle containing a

vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to a cell in the subject. Administration can be an *ex vivo* administration directly to a cell removed from a subject, such as any of the cells listed above, followed by replacement of the cell back into the subject, or administration can be *in vivo* administration to a cell in the subject. For *ex vivo* administration, cells are isolated from a subject by standard means according to the cell type and placed in appropriate culture medium, again according to cell type (*see, e.g.*, ATCC catalog). Viral particles are then contacted with the cells as described above, and the virus is allowed to transfect the cells. Cells can then be transplanted back into the subject's body, again by means standard for the cell type and tissue (*e. g.*, for neural cells, Dunnett, S.B. and Björklund, A., eds., *Transplantation: Neural Transplantation-A Practical Approach,* Oxford University Press, Oxford (1992)). If desired, prior to transplantation, the cells can be studied for degree of transfection by the virus, by known detection means and as described herein.

The present invention further provides a method of delivering a nucleic acid to a cell in a subject having neutralizing antibodies to AAV1-8 comprising administering to the subject an BAAV particle containing a vector comprising the nucleic acid, thereby delivering the nucleic acid to a cell in the subject. A subject that has neutralizing antibodies to AAV1-8 can readily be determined by any of several known means, such as contacting AAV1-8 protein(s) with an antibody-containing sample, such as blood, from a subject and detecting an antigen-antibody reaction in the sample. Delivery of the AAV1-8 particle can be by either *ex vivo* or *in vivo* administration as herein described. Thus, a subject who might have an adverse immunogenic reaction to a vector administered in an AAV2 viral particle can have a desired nucleic acid delivered using an AAV1-8 particle. This delivery system can be particularly useful for subjects who have received therapy utilizing AAV1-8 particles in the past and have developed antibodies to AAV1-8. An BAAV regimen can now be substituted to deliver the desired nucleic acid.

In any of the methods of delivering heterologous nucleic acids to a cell or subject described herein, the BAAV-conjugated nucleic acid or BAAV particle-conjugated nucleic acids described herein can be used.

In vivo administration to a human subject or an animal model can be by any of many standard means for administering viruses, depending upon the target organ, tissue or cell. Virus particles can be administered orally, parenterally (*e.g.*, intravenously), by intramuscular injection, intrarectally, by direct tissue or organ injection, by intraperitoneal injection, topically, transdermally, via aerosol delivery, via the mucosa or the like. Viral nucleic acids (non-encapsidated) can also be administered, *e.g.*, as a complex with cationic liposomes, or encapsulated in anionic liposomes. The present compositions can include various amounts of the selected viral particle or non-encapsidated viral nucleic acid in combination with a pharmaceutically acceptable carrier and, in addition, if desired, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. Parental administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Dosages will depend upon the mode of administration, the disease or condition to be treated, and the individual subject's condition, but will be that dosage typical for and used in administration of other AAV vectors, such as AAV2 vectors. Often a single dose can be sufficient; however, the dose can be repeated if desirable.

Administration methods can be used to treat brain disorders such as Parkinson's disease, Alzheimer's disease, and demyelination disease. Other diseases that can be treated by these methods include metabolic disorders such as , muscoloskeletal diseases, cardiovascular disease, cancer, and autoimmune disorders.

Administration of this recombinant BAAV virion to the cell can be accomplished by any means, including simply contacting the particle, optionally contained in a desired liquid such as tissue culture medium, or a buffered saline solution, with the cells. The virion can be allowed to remain in contact with the cells for any desired length of time, and typically the virion is administered and allowed to

remain indefinitely. For such *in vitro* methods, the virion can be administered to the cell by standard viral transduction methods, as known in the art and as exemplified herein. Titers of virus to administer can vary, particularly depending upon the cell type, but will be typical of that used for AAV transduction in general which is well known in the art. Additionally the titers used to transduce the particular cells in the present examples can be utilized.

The cells that can be transduced by the present recombinant BAAV virion can include any desired cell, such as the following cells and cells derived from the following tissues, human as well as other mammalian tissues, such as primate, horse, sheep, goat, pig, dog, rat, and mouse and avian species: Adipocytes, Adenocyte, Adrenal cortex, Amnion, Aorta, Ascites, Astrocyte, Bladder, Bone, Bone marrow, Brain, Breast, Bronchus, Cardiac muscle, Cecum, Cervix, Chorion, Cochlear, Colon, Conjunctiva, Connective tissue, Cornea, Dermis, Duodenum, Embryonic stem cells, Endometrium, Endothelium, Endothelial cells, Epithelial tissue, Epithelial cells, Epidermis, Esophagus, Eye, Fascia, Fibroblasts, Foreskin, Gastric, Glial cells, Glioblast, Gonad, Hepatic cells, Histocyte, Hair cells in the inner ear, Ileum, Intestine, small Intestine, Jejunum, Keratinocytes, Kidney, Larynx, Leukocytes, Lipocyte, Liver, Lung, Lymph node, Lymphoblast, Lymphocytes, Macrophages, Mammary alveolar nodule, Mammary gland, Mastocyte, Maxilla, Melanocytes, Mesenchymal, Monocytes, Mouth, Myelin, Myoblasts Nervous tissue, Neuroblast, Neurons, Neuroglia, Osteoblasts, Osteogenic cells, Ovary, Palate, Pancreas, Papilloma, Peritoneum, Pituicytes, Pharynx, Placenta, Plasma cells, Pleura, Prostate, Rectum, Salivary gland, Skeletal muscle, Skin, Smooth muscle, Somatic, Spleen, Squamous, Stem cells, Stomach, Submandibular gland, Submaxillary gland, Synoviocytes, Testis, Thymus, Thyroid, Trabeculae, Trachea, Turbinate, Umbilical cord, Ureter, Uterus, and vestibular hair cells.

The present invention provides recombinant vectors based on BAAV. Such vectors may be useful for transducing erythroid progenitor cells or cells resistant to transduction by other serotypes of AAV. These vectors may also be useful for transducing cells with a nucleic acid of interest in order to produce cell lines that could

be used to screen for agents that interact with the gene product of the nucleic acid of interest. In addition to transduction of other cell types, transduction of erythroid cells would be useful for the treatment of cancer and genetic diseases which can be corrected by bone marrow transplants using matched donors. Some examples of this type of treatment include, but are not limited to, the introduction of a therapeutic gene such as genes encoding interferons, interleukins, tumor necrosis factors, adenosine deaminase, cellular growth factors such as lymphokines, blood coagulation factors such as factor VIII and IX, cholesterol metabolism uptake and transport protein such as EpoE and LDL receptor, and antisense sequences to inhibit viral replication of, for example, hepatitis or HIV.

The present invention provides a vector comprising the BAAV virus as well as BAAV viral particles. While BAAV is similar to AAV1-8, the viruses are found herein to be physically and genetically distinct. These differences endow BAAV with some unique advantages, which better suit it as a vector for gene therapy.

Furthermore, as shown herein, BAAV capsid protein is distinct from AAV1-8 and AAAV capsid protein and exhibits different tissue tropism. AAV1-8 and BAAV likely utilize distinct cellular receptors. AAV1-8 and BAAV are serologically distinct and humans are not reported to have neutralizing antibodies to BAAV,thus in a gene therapy or gene transfer application, BAAV would allow for transduction of a patient who already possess neutralizing antibodies to AAV1-8 either as a result of natural immunological defense or from prior exposure to AAV1-8 vectors.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

To understand the nature of BAAV virus and to determine its usefulness as a vector for gene transfer, it was cloned and sequenced.

Materials and Methods

Cell culture and virus propagation. 293T and COS cells were maintained in DMEM, supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, and 0.1mg/ml streptomycin. Cancer cell lines indicated in Fig. 1 were cultured in RPMI medium supplemented with 5% FBS, 2mM L-glutamine, 100U/ml penicillin, and 0.1mg/ml streptomycin. MDBK cells were propagated in DMEM supplemented with 5% horse serum, 2mM L-glutamine, 100U/ml penicillin, and 0.1mg/ml streptomycin. Cells were maintained at 37°C in a 5% CO2 humidified atmosphere.

Bovine Adenovirus Type 1 (ATCC VR-313) and Bovine Adenovirus Type 2 (ATCC VR-313) obtained from ATCC are reported by ATCC to be contaminated with AAV. For virus propagation, MDBK cells were infected with ATCC VR-313 or ATCC VR-314 and cultured for 5 days. At this time, first signs of an adenovirus induced cytopathic effect was observed.

Viral DNA isolation, cloning and sequencing.

Viral DNA was isolated from the Bovine Adenovirus Type 1 (ATCC VR-313) and Bovine Adenovirus Type 2 (ATCC VR-313) infected MDBK cells using the High Pure Viral Nucleic Acid Kit (Roche). These DNA samples were assayed for AAV contamination by PCR using the GC Rich PCR Kit (Roche) as described in Katano and Chiorini, 2003, Identification of Adeno-associated virus contamination by PCR (submitted to J Virol). Briefly, this method detects the presence of AAV DNA by PCR using degenerative PCR primers, which were shown to amplify a fragment containing sequences of the rep and vp ORF of all known AAV serotypes. PCR using DNA isolated from ATCC VR-313 and ATCC VR-314 as template resulted in the generation of a 1.4kb amplification product, which was subsequently cloned using the TOPO TA Cloning KIT (Invitrogen) and sequenced with an ABI Prism 3100 Genetic Analyzer (ABI) and FS dye-terminator chemistry (ABI). The obtained sequences showed homology to AAV5 rep ORF and AAV4 cap ORF but were not identical to any known AAV. This result demonstrated that ATCC VR-313 and ATCC VR-314 contained contaminations of an unknown AAV serotype, termed subsequently bovine adeno-associated virus (BAAV). The obtained sequence of BAAV was used to generate PCR

primers that bind in the BAAV rep ORF in (-) orientation and in the vp ORF in (+) orientation. PCR using these primers and extrachromosomal DNA of ATCC VR-313 infected MDBK cells (isolated using the Qiagen Mini Prep Kit) resulted in amplification of a BAAV fragment spanning from the vp ORF through the ITR to the rep ORF. The PCR amplification products were subsequently cloned using the TA Cloning KIT (Invitrogen) and sequenced with an ABI Prism 3100 Genetic Analyzer (ABI) and FS dye-terminator chemistry (ABI). ITRs of 2 clones were sequenced by isothermal non-cycling sequencing chemistry using radiolabeled dCTP (Epicentre). For the generation of recombinant particles, a BAAV packaging plasmid was constructed by PCR amplifying a BAAV fragment containing the complete ORF of rep and vp using DNA isolated from ATCC VR-313 and ATCC VR-314 samples as template and inserting this fragment into an expression plasmid under the control of a MMTV promoter resulting in the plasmid pMMTV-BAAV#1-200. 10 clones were sequenced. The plasmids were assayed for the ability to generate recombinant BAAV particles by transfecting 293 T cells with an AAV5-NLS-GFP vector plasmid, pMMTV-BAAV and p449b helper plasmid. 2 days after transfection, cells were lysed by 3 freeze thaw cycles. Cleared lysate was used to infect Cos cells. 2 days after infection, cells were assayed for GFP expression by fluorescent microscopy. pMMTV-BAAV#47 generated highest titers of recombinant BAAV but diverged from the BAAV consensus sequence by 1 nucleotide change. The sequence of pMMTV-BAAV#47 was changed to the consensus sequence using the Quik Change Kit (Clontech) and named pMMTV-BAAV.

Sequence analysis. DNA and protein sequence alignments were performed using the Clustal W multiple sequence alignment tool of the Biology Workbench web based software (SDSC), MacVector 7 (Oxford Molecular). Promoters, transcription initiation and splice sites were predicted using the Neural Network Promoter Prediction web paged software (BDGP). The genome of BAAV is 4,694 nucleotides in length and has similar organization with that of other AAVs (Fig.1A). The entire genome of BAAV displays 54-79% identity at the nucleotide level with the other known AAVs. Highest homology was observed with AAV5 (79%), lowest homology to BAAV showed

AAAV with 54% (Fig.1B). The BAAV genome has inverted terminal repeats of 150 nucleotides with are forming the characteristic T-shaped palindromic structure. The putative Rep-binding element (RBE) consists of a tandem $(GAGY)_4$ repeat, and the putative terminal resolution site (*trs*), AGTGTGG (Fig.2). The BAAV ITR is greater than 95% identical to AAV5 and contains a trs that is identical to AAV5 as well as a conserved RRE. The Rep ORF of BAAV displays 48-89 % identity at the amino acid level with the other AAVs, with most of the diversity clustered at the amino termini. A surprisingly high homology of 89 % was found with AAV5 (Fig.3A and 3C). Comparison of the capsid proteins of BAAV and the primate dependoviruses revealed 55-76 % identity with other known AAVs (Fig.3B and 3D). AAV4 showed the highest homology to BAAV with 76% while AAAV was most divergent with 55% identity to BAAV Vp1. Divergent regions in the capsid ORF are clustered in surface exposed loops.

Generation of recombinant virus

The high homology between the BAAV and AAV5 ITR and Rep amino acid sequence led to the assumption that BAAV can replicate and package AAV5 ITR containing vectors. This assumption was confirmed in initial experiments; AAV5 ITR containing vector plasmids containing a lacZ expression cassette were replicated and packaged with AAV5 or BAAV packaging plasmids with equal efficiency. Therefore, AAV5 ITR containing vector plasmids were used for all subsequent studies to produce recombinant BAAV.

Recombinant BAAV was generated by transfecting 293 T cells with AAV5 vector, BAAV packaging and Ad helper plasmids. 3 confluent T175 flasks of 293T cells were harvested, resuspended in 100ml DMEM 10%FCS, seeded in 10 150mm plates and incubated at 37 °C, 5% CO_2 until cells are 80% confluent (typically 48h). Cells were transfected with 15µg ~~pAAV5-NLS-GFP~~ or pAAV5-RnlacZ, 15µg pMMTV-BAAV and 30µg p449B. 48h after transfection, cells were harvested, washed with PBS and resuspended in 11ml TD buffer (0.14 M NaCl, 5.0 mM KCl, 0.7 mM K_2HPO_4, 25.0 mM Tris, pH7.4. Cells were lyzed by 3 freeze thaw cycles and incubated for 30 minutes at 37°C after adding benzonase to a final concentration of 20 U/ml and sodium

deoxycholate (final concentration of 0.5%). After adding 0.55g CsCl/ ml the lysate was fractionated using density gradient centrifugation in a SW41 rotor for 48h at 38000 rpm. The gradients were harvested in 0.5 ml aliquots. Aliquots were assayed for infectivity and particle titer were determined by real time PCR using primers binding in the promoter region of the vectors.

Determination of tissue tropism

Transduction efficiency of recombinant BAAV vector containing an expression cassette for beta-galactosidase (rBAAV-RnlacZ) was analyzed in 60 cancer cell lines (NCI cancer cell panel). Cells were infected with an MOI of 10 with Ad5 and 2h later with rBAAV-RnlacZ in 10 fold serial dilutions ranging from 10^2 to 10^9 particles/well. 48h after infection, cells were fixed and stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl–β-D-galactopyranoside (X-Gal) (Gold BioTechnology, Inc. St. Louis Mo). Transduced cells were visually counted using a light microscope. GFP expressing cells were detected using fluorescent microscopy. Results were used to calculate the number of transduced cells for 10^9 particles (Fig.4). rBAAV efficient transduction of a wide variety of tumor cells including cells of CNS, colon, prostate, renal, breast and ovarian lineage. It is therefore a potent vector for gene transfer in a wide variety of gene therapy applications.

Neutralizing antibody assay

AAV isolates that are serological distinct can be distinguished by neutralization assays and are often referred to as AAV serotypes. We analyzed if BAAV elicts a BAAV specific immune response in mice that did not cross react with other AAV serotypes. Balbc mice were injected with 10^{10} particles of BAAV-RnlacZ, AAV2-RnlacZ, AAV4-RnlacZ and AAV5-RnlacZ. 4 weeks after infection serum of the infected animals was assayed in a neutralizing antibody assay.

Exponentially growing COS cells (7×10^3) were plated in a density of 7×10^3/ well in a flat-bottomed 96-well plate. 24h after seeding, cells were infected with wild-type adenovirus with a multiplicity of infection [MOI]of 10 for 1 h. Heat inactivated sera of rAAV2, rAAV4, rAAV5 and rBAAV infected mice were serial diluted from 1:200 to

a 1:12800 in RPMI containing 1% fatal calf serum (FCS). 40 transducing units of BAAV-RnlacZ (Fig.5A) or AAV4-RnlacZ (Fig.5B) (were added to the diluted sera and incubated for 1h at 37°C . Subsequently, the virus/sera mixture was added to COS cells. 24h after rAAV infection, cells were assayed for beta-galactosidase expression X-Gal staining (Gold BioTechnology, Inc. St. Louis Mo). Transduced cells were visually counted using a light microscope. Neutralizing titers of the sera ware calculated as the highest dilution that inhibited 50% of transduction. Any serum dilution in which more than 70% reduction of positive cells compared with serum-free media remained was considered to be positive for neutralizing activity. All samples were assayed in duplicate or triplicate.

rBAAV elicted a unique immune response in mice that efficiently neutralized rBAAV, bud did not cross-react with rAAV4. Sera of rAAV2, rAAV4 and rAAV5 infected mice did not neutralize rBAAV. These results demonstrate that BAAV is a new AAV serotype.

Transduction of submandibular glands in vivo

10^{10} particles of AAV2-lacZ and BAAV-lacZ were injected into submandibular glands of BALB/c mice by retrograde ductal instillation as described earlier (Yamano et al., 2002). 4 weeks after infection, blood was collected form experimental animals by retro-orbital plexus bleed. Submandibular glands were excised, homogenized and lyzed in 500 µL of Galact-light lysis solution (100 mM potassium phosphate (pH7.8), 0.2% TritonX-100) (Applied biosystems). PMSF and leupeptin were added to a final concentration of 0.2 mM and 5 µg/mL respectively. The lysate was cleared by centrifugation at 10,000 rpm for 5min. Genomic DNA was extracted from a 100-µL aliquot using the Wizard DNA extraction kit (Promega) according to the manufacturer's instructions. DNA concentrations were determined by spectrophotometry. Detection and quantification of genome copies of the AAV vectors was done by quantitative real time PCR using a TaqMan system (Applied Biosystems) with probes specific to the RSV promoter as described earlier ((Yamano et al., 2002)). Protein concentration of the lysates was determined using the BCA protein assay kit (Pierce) and ß-Gal expression was quantified with a ß-Gal ELISA kit (Roche Molecular

Biochemicals). The ß-Gal levels were normalized for total protein concentration and expressed as picograms of ß-Gal per milligram of protein.

Recombinant BAAV was about ten fold more efficient than rAAV2 in the transduction of submandibular glands and expressing a gene of interest, demonstrating the feasibility of rBAAV to be used as a vector for gene therapy applications (Fig.6A and Fig.6B).

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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What is claimed is:

1. A nucleic acid vector comprising a pair of bovine adeno-associated virus (BAAV) inverted terminal repeats and a promoter between the inverted terminal repeats.

2. The vector of claim 1, wherein the promoter is an adeno-associated virus (AAV) promoter p5.

3. The vector of claim 1, wherein the p5 promoter is BAAV p5 promoter.

4. The vector of claim 1, further comprising an exogenous nucleic acid functionally linked to the promoter.

5. The vector of claim 1 encapsidated in an adeno-associated virus particle.

6. The particle of claim 5, wherein the particle is a BAAV particle.

7. The particle of claim 5, wherein the particle is an AAV1 particle, an AAV2 particle, an AAV3 particle, an AAV4 particle, an AAV5, an AAV6, an AAV7, an AAV8 or an avian adeno-associated virus (AAAV) particle.

8. The particle of claim 5, wherein the particle is parvovirus particle.

9. The particle of claim 5, wherein the particle is dependent parvovirus particle.

10. The particle of claim 5, wherein the particle is adenovirus particle.

11. A recombinant BAAV virion containing a vector comprising a pair of AAV1, AAV2, AAV3, AAV4, AAV5, ~~AAV6, AAV7~~, AAV8, AAAV or BAAV inverted terminal repeats.

12. The virion of claim 8, wherein the vector further comprises an exogenous nucleic acid inserted between the inverted terminal repeats.

13. An isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:1.

14. An isolated nucleic acid consisting essentially of the nucleotide sequence set forth in SEQ ID NO:1.

15. An isolated nucleic acid that selectively hybridizes with the nucleic acid of claim 14.

16. An isolated nucleic acid encoding a BAAV Rep protein.

17. The nucleic acid of claim 16, wherein the BAAV Rep protein has the nucleic acid sequence set forth in SEQ ID NO:2.

18. The nucleic acid of claim 16, wherein the BAAV Rep protein has the amino acid sequence set forth in SEQ ID NO:3.

19. The nucleic acid of claim 16, wherein the BAAV Rep protein has the amino acid sequence set forth in SEQ ID NO:5.

20. The nucleic acid of claim 16, wherein the BAAV Rep protein has the nucleic acid sequence set forth in SEQ ID NO:4.

21. An isolated BAAV Rep protein.

22. The isolated BAAV Rep protein of claim 21, having the nucleic acid sequence set forth in SEQ ID NO:2, or a unique fragment thereof.

23. The isolated BAAV Rep protein of claim 21, having the amino acid sequence set forth in SEQ ID NO:3, or a unique fragment thereof.

24. An isolated antibody that specifically binds the protein of claim 21.

25. An isolated BAAV capsid protein.

26. The isolated BAAV capsid protein of claim 25 having the amino acid sequence set forth in SEQ ID NO:9.

27. The isolated BAAV capsid protein of claim 25 having the amino acid sequence set forth in SEQ ID NO:9, wherein the sequence comprises modifications in order to alter the tropism of the virus.

28. The isolated BAAV capsid protein of claim 25 having the amino acid sequence set forth in SEQ ID NO:9, wherein the sequence comprises modifications in order to develop a host immune response to the epitope.

29. The isolated BAAV capsid protein of claim 25 having the amino acid sequence set forth in SEQ ID NO:9, wherein the sequence comprises modifications in order to block an immune response directed at the virus.

30. An isolated antibody that specifically binds the protein of claim 25.

31. The isolated BAAV capsid protein of claim 25, having the amino acid sequence set forth in SEQ ID NO:7.

32. An isolated antibody that specifically binds the protein of claim 31.

33. The isolated BAAV capsid protein of claim 25, having the amino acid sequence set forth in SEQ ID NO:11.

34. An isolated antibody that specifically binds the protein of claim 33.

35. An isolated nucleic acid encoding the protein of claim 26.

36. The nucleic acid of claim 35, having the nucleic acid sequence set forth in SEQ ID NO:6.

37. The nucleic acid of claim 35, having the nucleic acid sequence set forth in SEQ ID NO:8.

38. The nucleic acid of claim 35, having the nucleic acid sequence set forth in SEQ ID NO:10.

39. An isolated nucleic acid that selectively hybridizes with the nucleic acid of claim 35.

40. A BAAV particle comprising a capsid protein consisting essentially of the amino acid sequence set forth in SEQ ID NO:7.

41. An isolated nucleic acid comprising a BAAV p5 promoter.

42. A method of screening a cell for infectivity by BAAV, comprising contacting the cell with BAAV and detecting the presence of BAAV in the cells.

43. A method of determining the suitability of a BAAV vector for administration to a ~~subject~~, ~~comprising contacting an antibody-containing sample from~~ the subject with an antigenic fragment of a protein of claim 25 and detecting an antibody-antigen reaction in the sample, the presence of a neutralizing reaction indicating the BAAV vector to be unsuitable for use in the subject.

44. A method of determining the presence in a subject of a BAAV-specific antibody comprising, contacting an antibody-containing sample from the subject with an antigenic fragment of the protein of claim 25 and detecting an antibody-antigen reaction in the sample, the presence of a reaction indicating the presence of a BAAV-specific antibody in the subject.

45. A method of delivering a nucleic acid to a cell, comprising administering to the cell a BAAV particle containing a vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to the cell.

46. The method of claim 45, wherein the AAV inverted terminal repeats are BAAV inverted terminal repeats.

47. A method of delivering a nucleic acid to a subject comprising administering to a cell from the subject a BAAV particle comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, and returning the cell to the subject, thereby delivering the nucleic acid to the subject.

48. A method of delivering a nucleic acid to a cell in a subject comprising administering to the subject a BAAV particle comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to a cell in the subject.

49. A method of delivering a nucleic acid to a cell in a subject having antibodies to AAV2 comprising administering to the subject a BAAV particle comprising the nucleic acid, thereby delivering the nucleic acid to a cell in the subject.

50. An isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:12.

51. An isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID

NO: 12.

52. A vector system for producing infectious virus particles having a characteristic of BAAV comprising: at least one vector comprising a nucleic acid selected from the group consisting of a pair of BAAV inverted terminal repeats, a nucleic acid encoding a BAAV capsid protein, and a nucleic acid encoding a BAAV Rep protein.

53. The vector system of claim 52, comprising two vectors.

54. The vector system of claim 53, wherein the first vector comprises a nucleic acid encoding a BAAV Rep protein and the second vector comprises a pair of BAAV inverted terminal repeats.

55. The vector system of claim 53, wherein the first vector comprises a nucleic acid encoding a BAAV capsid protein and a nucleic acid encoding a BAAV Rep protein and the second vector comprises a pair of BAAV inverted terminal repeats.

56. The vector system of claim 53, wherein the first vector comprises a nucleic acid encoding a BAAV capsid protein and the second vector comprises a pair of AAV inverted terminal repeats.

57. The vector system of claim 56, wherein the second vector comprises a pair of AAV1 inverted terminal repeats.

58. The vector system of claim 56, wherein the second vector comprises a pair of AAV2 inverted terminal repeats.

59. The vector system of claim 56, wherein the second vector comprises a pair of AAV3 inverted terminal repeats.

60. The vector system of claim 56, wherein the second vector comprises a pair of

AAV4 inverted terminal repeats.

61. The vector system of claim 56, wherein the second vector comprises a pair of BAAV inverted terminal repeats.

62. The vector system of claim 56, wherein the second vector comprises a pair of AAV6 inverted terminal repeats.

63. The vector system of claim 56, wherein the first vector further comprises a nucleic acid encoding an AAV1 Rep protein.

64. The vector system of claim 56, wherein the first vector further comprises a nucleic acid encoding an AAV2 Rep protein.

65. The vector system of claim 56, wherein the first vector further comprises a nucleic acid encoding an AAV3 Rep protein.

66. The vector system of claim 56, wherein the first vector further comprises a nucleic acid encoding an AAV4 Rep protein.

67. The vector system of claim 56, wherein the first vector further comprises a nucleic acid encoding a BAAV Rep protein.

68. The vector system of claim 56, wherein the first vector further comprises a nucleic acid encoding an AAV6 Rep protein.

69. The vector system ~~of claim 55, wherein~~ the first vector comprises a nucleic acid encoding an AAV capsid protein and the second vector comprises a pair of BAAV inverted terminal repeats.

70. The vector system of claim 69, wherein the first vector comprises a nucleic acid

encoding an AAV1 capsid protein.

71. The vector system of claim 69, wherein the first vector comprises a nucleic acid encoding an AAV2 capsid protein.

72. The vector system of claim 69, wherein the first vector comprises a nucleic acid encoding an AAV3 capsid protein.

73. The vector system of claim 69, wherein the first vector comprises a nucleic acid encoding an AAV4 capsid protein.

74. The vector system of claim 69, wherein the first vector comprises a nucleic acid encoding a BAAV capsid protein.

75. The vector system of claim 69, wherein the first vector comprises a nucleic acid encoding an AAV6 capsid protein.

76. The vector system of any of claims 69 to 75, wherein the first vector further comprises a nucleic acid encoding a BAAV Rep protein.

77. The vector system of any of claims 69 to 75, wherein the second vector further comprises a promoter between the inverted terminal repeats.

78. The vector system of claim 77, wherein the promoter is functionally linked to an exogenous nucleic acid.

79. A vector comprising a pair of AAV inverted terminal repeats, a nucleic acid encoding a BAAV capsid protein and a nucleic acid encoding an AAV Rep protein.

80. The vector of claim 79, encapsidated in a dependent parvovirus particle.

81. The vector of claim 80, wherein the particle which encapsidates the vector is an AAV1 particle, an AAV2 particle, an AAV3 particle, an AAV4 particle, an AAV5 particle, an AAV6 particle, an AAV7 particle, an AAV8 particle , an AAAV particle or a BAAV particle.

ABSTRACT OF THE DISCLOSURE

The present invention provides an bovine adeno-associated virus (BAAV) virus and vectors and particles derived therefrom. In addition, the present invention provides methods of delivering a nucleic acid to a cell using the BAAV vectors and particles.

SEQUENCE LISTING

1) SEQ ID NO:1 (BAAV complete genome)

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3) SEQ ID NO:3 (BAAV Rep78)
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4) SEQ ID NO:4 (BAAV rep52 ORF)
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8) SEQ ID NO: 8 (BAAV vp2 ORF)

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GAGGAACTCTCAACCAGGGCAATTCAGCCACCAACTTTGCCAAGCT
GACCAAAACAAACTTTTCTGGCTACCGCAAAAACTGGCTCCCGGGG
CCCATGATGAAGCAGCAGAGATTCTCCAAGACTGCCAGTCAAAACT
ACAAGATTCCCCAGGGAAGAAACAACAGTCTGCTCCATTATGAGAC
CAGAACTACCCTCGACGGAAGATGGAGCAATTTTGCCCCGGGAACG
GCCATGGCAACCGCAGCCAACGACGCCACCGACTTCTCTCAGGCCC
AGCTCATCTTTGCGGGGCCCAACATCACCGGCAACACCACCACAGAT
GCCAATAACCTGATGTTCACTTCAGAAGATGAACTTAGGGCCACCAA
CCCCCGGGACACTGACCTGTTTGGCCACCTGGCAACCAACCAGCAA
AACGCCACCACCGTTCCTACCGTAGACGACGTGGACGGAGTCGGCG
TGTACCCGGGAATGGTGTGGCAGGACAGAGACATTTACTACCAAGG
GCCCATTTGGGCCAAAATTCCACACACGGATGGACACTTTCACCCGT
CTCCTCTCATTGGCGGATTTGGACTGAAAAGCCCGCCTCCACAAATA
TTCATCAAAAACACTCCTGTACCCGCCAATCCCGCAACGACCTTCTC
TCCGGCCAGAATCAACAGCTTCATCACCCAGTACAGCACCGGACAG
GTGGCTGTCAAAATAGAATGGGAAATCCAGAAGGAGCGGTCCAAGA
GATGGAACCCAGAGGTCCAGTTCACGTCCAACTACGGAGCACAGGA
CTCGCTTCTCTGGGCTCCCGACAACGCCGGAGCCTACAAAGAGCCCA
GGGCCATT[redacted]CACCAACCACCTCTAG

9) SEQ ID NO:9 (BAAV Vp2)

TAPAAKKRPLEQSPQEPDSSSGVGKKGKQPARKRLNFDDEPGAGDGPP
PEGPSSGAMSTETEMRAAAGGNGGDAGQGAEGVGNASGDWHCDSTW
SESHVTTTSTRTWVLPTYNNHLYLRLGSSNASDTFNGFSTPWGYFDFNR

FHCHFSPRDWQRLINNHWGLRPKSMQVRIFNIQVKEVTTSNGETTVSNN
LTSTVQIFADSTYELPYVMDAGQEGSLPPFPNDVFMVPQYGYCGLVTG
GSSQNQTDRNAFYCLEYFPSQMLRTGNNFEMVYKFENVPFHSMYAHS
QSLDRLMNPLLDQYLWELQSTTSGGTLNQGNSATNFAKLTKTNFSGYR
KNWLPGPMMKQQRFSKTASQNYKIPQGRNNSLLHYETRTTLDGRWSN
FAPGTAMATAANDATDFSQAQLIFAGPNITGNTTTDANNLMFTSEDELR
ATNPRDTDLFGHLATNQQNATTVPTVDDVDGVGVYPGMVWQDRDIY
YQGPIWAKIPHTDGHFHPSPLIGGFGLKSPPPQIFIKNTPVPANPATTFSPA
RINSFITQYSTGQVAVKIEWEIQKERSKRWNPEVQFTSNYGAQDSLLWA
PDNAGAYKEPRAIGSRYLTNHL

10) SEQ ID NO:10 (BAAV vp3 ORF)

ATGCGTGCAGCAGCTGGCGGAAATGGTGGCGATGCGGGACAAGGTG
CCGAGGGAGTGGGTAATGCCTCCGGTGATTGGCATTGCGATTCCACT
TGGTCAGAGAGCCACGTCACCACCACCTCAACCCGCACCTGGGTCCT
GCCGACCTACAACAACCACCTGTACCTGCGGCTCGGCTCGAGCAAC
GCCAGCGACACCTTCAACGGATTCTCCACCCCCTGGGGATACTTTGA
CTTTAACCGCTTCCACTGCCACTTCTCGCCAAGAGACTGGCAAAGGC
TCATCAACAACCACTGGGGACTGCGCCCCAAAAGCATGCAAGTCCG
CATCTTCAACATCCAAGTTAAGGAGGTCACGACGTCTAACGGGGAG
ACGACCGTATCCAACAACCTCACCAGCACGGTCCAGATCTTTGCGGA
CAGCACGTACGAGCTCCCGTACGTGATGGATGCAGGTCAGGAGGGC
AGCTTGCCTCCTTTCCCCAACGACGTGTTCATGGTGCCTCAGTACGG
GTACTGCGGACTGGTAACCGGAGGCAGCTCTCAAAACCAGACAGAC
AGAAATGCCTTCTACTGTCTGGAGTACTTTCCCAGCCAGATGCTGAG
AACCGGAAACAACTTTGAGATGGTGTACAAGTTTGAAAACGTGCCC
TTCCACTCCATGTACGCTCACAGCCAGAGCCTGGATAGGCTGATGAA
CCCGCTGCTGGACCAGTACCTGTGGGAGCTCCAGTCTACCACCTCTG
GAGGAACTCTCAACCAGGGCAATTCAGCCACCAACTTTGCCAAGCT
GACCAAAACAAACTTTTCTGGCTACCGCAAAAACTGGCTCCCGGGG
CCCATGATGAAGCAGCAGAGATTCTCCAAGACTGCCAGTCAAAACT
ACAAGATTCCCCAGGGAAGAAACAACAGTCTGCTCCATTATGAGAC
CAGAACTACCCTCGACGGAAGATGGAGCAATTTTGCCCCGGGAACG
GCCATGGCAACCGCAGCCAACGACGCCACCGACTTCTCTCAGGCCC
AGCTCATCTTTGCGGGGCCCAACATCACCGGCAACACCACCACAGAT
GCCAATAACCTGATGTTCACTTCAGAAGATGAACTTAGGGCCACCAA
CCCCCGGGACACTGACCTGTTTGGCCACCTGGCAACCAACCAGCAA
AACGCCACCACCGTTCCTACCGTAGACGACGTGGACGGAGTCGGCG
TGTACCCGGGAATGGTGTGGCAGGACAGAGACATTTACTACCAAGG
GCCCATTTGGGCCAAAATTCCACACACGGATGGACACTTTCACCCGT
CTCCTCTCATTGGCGGATTTGGACTGAAAAGCCCGCCTCCACAAATA
TTCATCAAAAACACTCCTGTACCCGCCAATCCCGCAACGACCTTCTC
TCCGGCCAGAATCAACAGCTTCATCACCCAGTACAGCACCGGACAG
GTGGCTGTCAAAATAGAATGGGAAATCCAGAAGGAGCGGTCCAAGA
GATGGAACCCAGAGGTCCAGTTCACGTCCAACTACGGAGCACAGGA

CTCGCTTCTCTGGGCTCCCGACAACGCCGGAGCCTACAAAGAGCCCA
GGGCCATTGGATCCCGATACCTCACCAACCACCTCTAG

11) SEQ ID NO:11 (BAAV Vp3)
MRAAAGGNGGDAGQGAEGVGNASGDWHCDSTWSESHVTTTSTRTWV
LPTYNNHLYLRLGSSNASDTFNGFSTPWGYFDFNRFHCHFSPRDWQRLI
NNHWGLRPKSMQVRIFNIQVKEVTTSNGETTVSNNLTSTVQIFADSTYE
LPYVMDAGQEGSLPPFPNDVFMVPQYGYCGLVTGGSSQNQTDRNAFY
CLEYFPSQMLRTGNNFEMVYKFENVPFHSMYAHSQSLDRLMNPLLDQ
YLWELQSTTSGGTLNQGNSATNFAKLTKTNFSGYRKNWLPGPMMKQQ
RFSKTASQNYKIPQGRNNSLLHYETRTTLDGRWSNFAPGTAMATAAND
ATDFSQAQLIFAGPNITGNTTTDANNLMFTSEDELRATNPRDTDLFGHL
ATNQQNATTVPTVDDVDGVGVYPGMVWQDRDIYYQGPIWAKIPHTDG
HFHPSPLIGGFGLKSPPPQIFIKNTPVPANPATTFSPARINSFITQYSTGQV
AVKIEWEIQKERSKRWNPEVQFTSNYGAQDSLLWAPDNAGAYKEPRAI
GSRYLTNHL*

12) SEQ ID NO:12 (ITR)
GTGGCACTCCCCCCCCTGTCGCGTTCGCTCGTTCGCTGGCTCGATTGG
GGGGGTGGCAGCTCAAAGAGCTGCCAGACGACGGCCCTCTGGGCCG
TCGCCCCCCCCAATCGAGCCAGCGAACGAGCGAACGCGACAGGGGGG
GGAGTGCCAC

13) SEQ ID NO:13 (D-region)
CTCTAGCAAGGGGGTTTTGT

14) SEQ ID NO:14 (TRS)
AGTGTGG

15) SEQ ID NO:15 (BAAV p5 promoter)
AGGTGGTGATGTCATTGTTGATGTCATTATAGTTGTCACGCGATAGT
TAATGATTAACAGTCATGTGATGTGTGTTATCCAATAGGATGAAAGC
GCGCGAATGAGATCTCGCGAGACTTCCGGGGTATAAAAGGGGTGAG
TGAACGAGCCCGCCGCCA

16) SEQ ID NO:16 (BAAV p19 promoter)
GGTGGATTCTGGGTATATTCCCGCCTACCTGCTGCCGAAGGTCCAAC
CAGAGCTTCAGTGGGCGTGGACTAACCTCGAAGAGTATAAATTGGC
CGCCCTCAATCTGGAGGAC

17) SEQ ID NO:17 (BAAV p40 promoter)
AGTCAAAGACTTTTTTGCTTGGGCAAAGGTCAACCAGGTGCCGGTGA
CTCACGAGTTTATGGTTCCCAAGAAAGTGGCGGGAACTGAGAGGGC
GGAGACTTCTAGAAAACGCCCACTGGATGACGTCACCAATACCAAC
TATAAAAGTCCGGAGAAGCGGGCCCGGCTC

Formatted Alignments of AAV Genomes

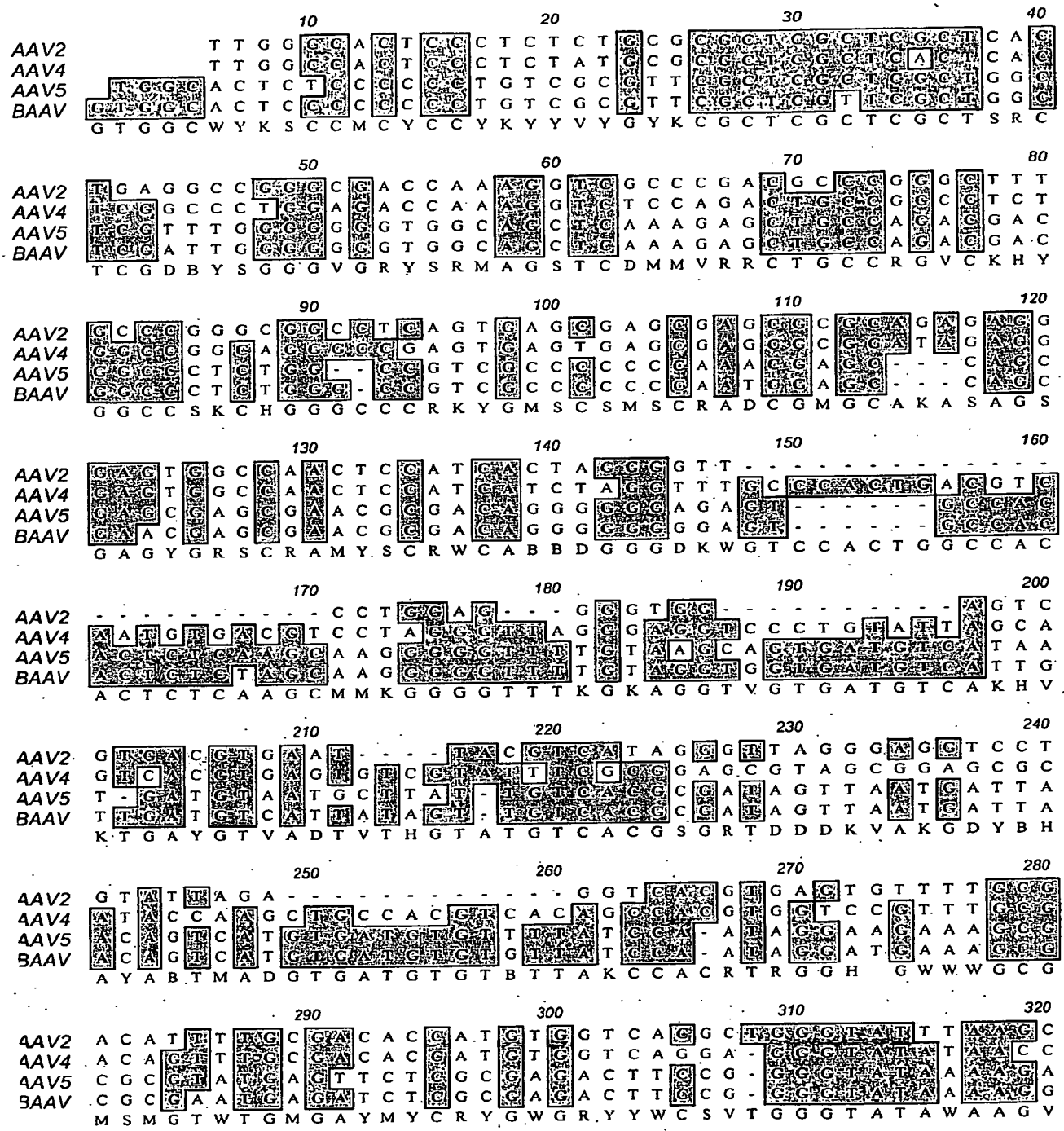


FIG. 1A

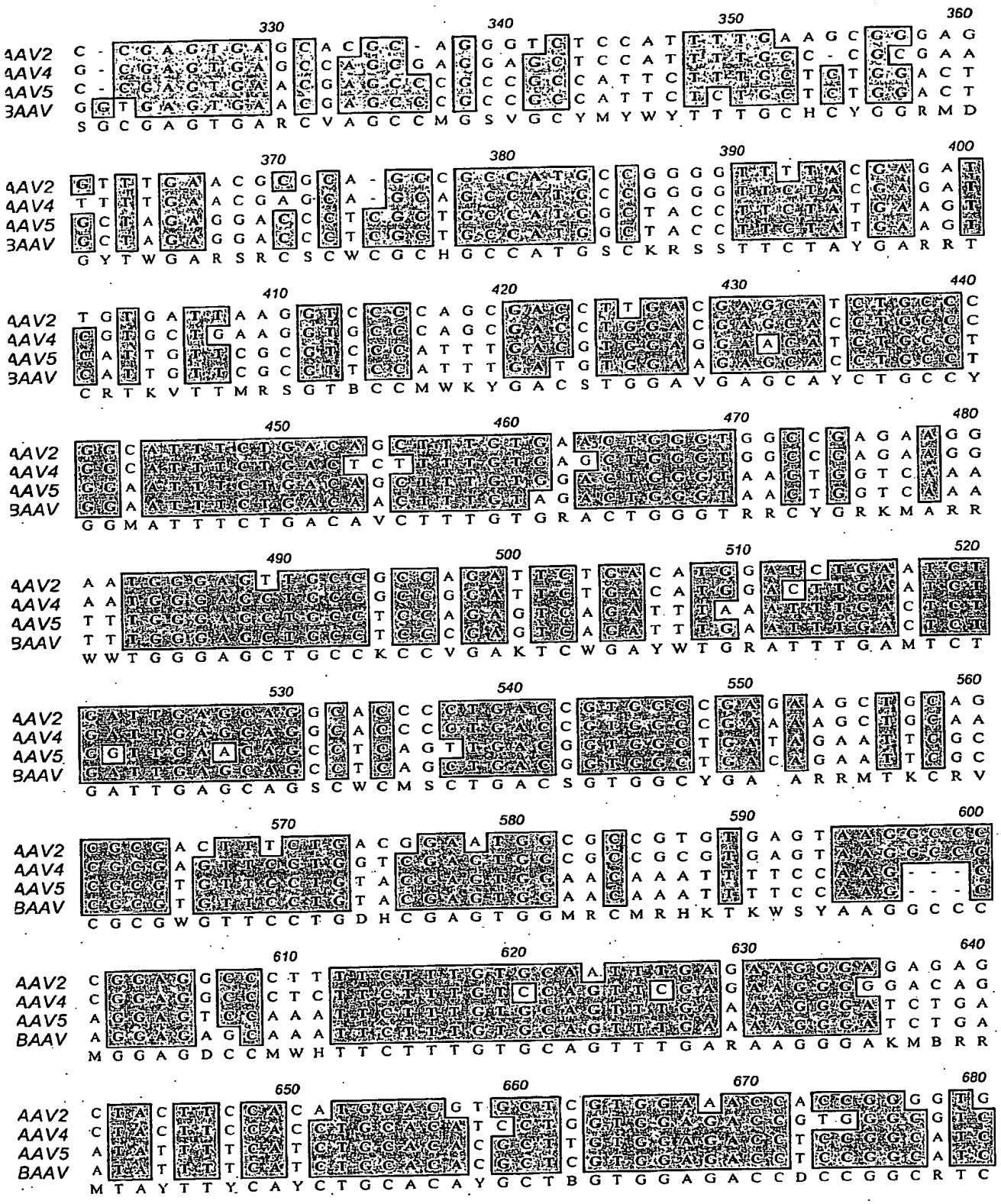


FIG. 1A

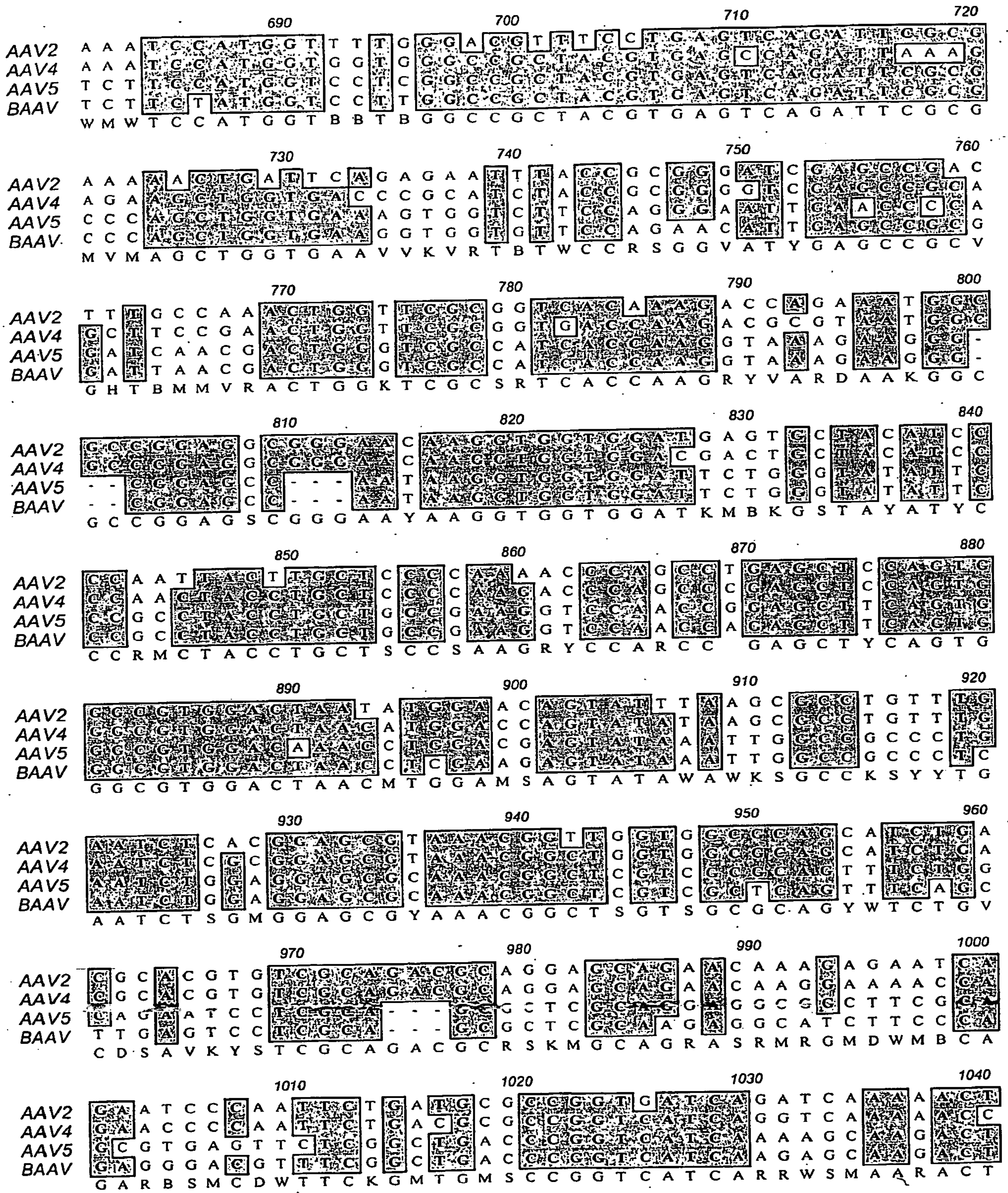

AAV2
AAV4
AAV5
BAAV
690
700
710
720
730
740
750
760
770
780
790
800
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970
980
990
1000
1010
1020
1030
1040

FIG. 1A

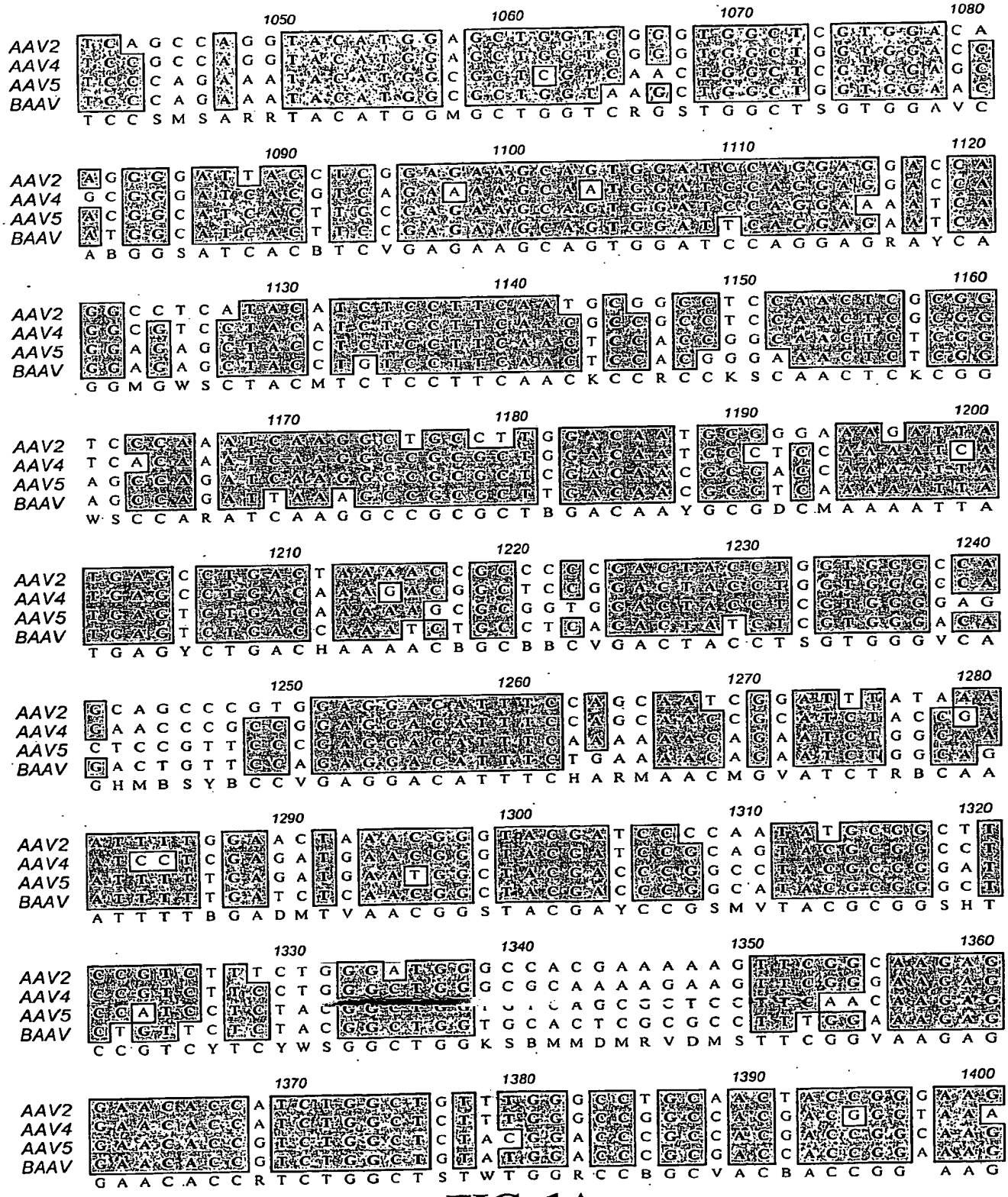

1050
1060
1070
1080
AAV2
AAV4
AAV5
BAAV
1090
1100
1110
1120
1130
1140
1150
1160
1170
1180
1190
1200
1210
1220
1230
1240
1250
1260
1270
1280
1290
1300
1310
1320
1330
1340
1350
1360
1370
1380
1390
1400

FIG. 1A

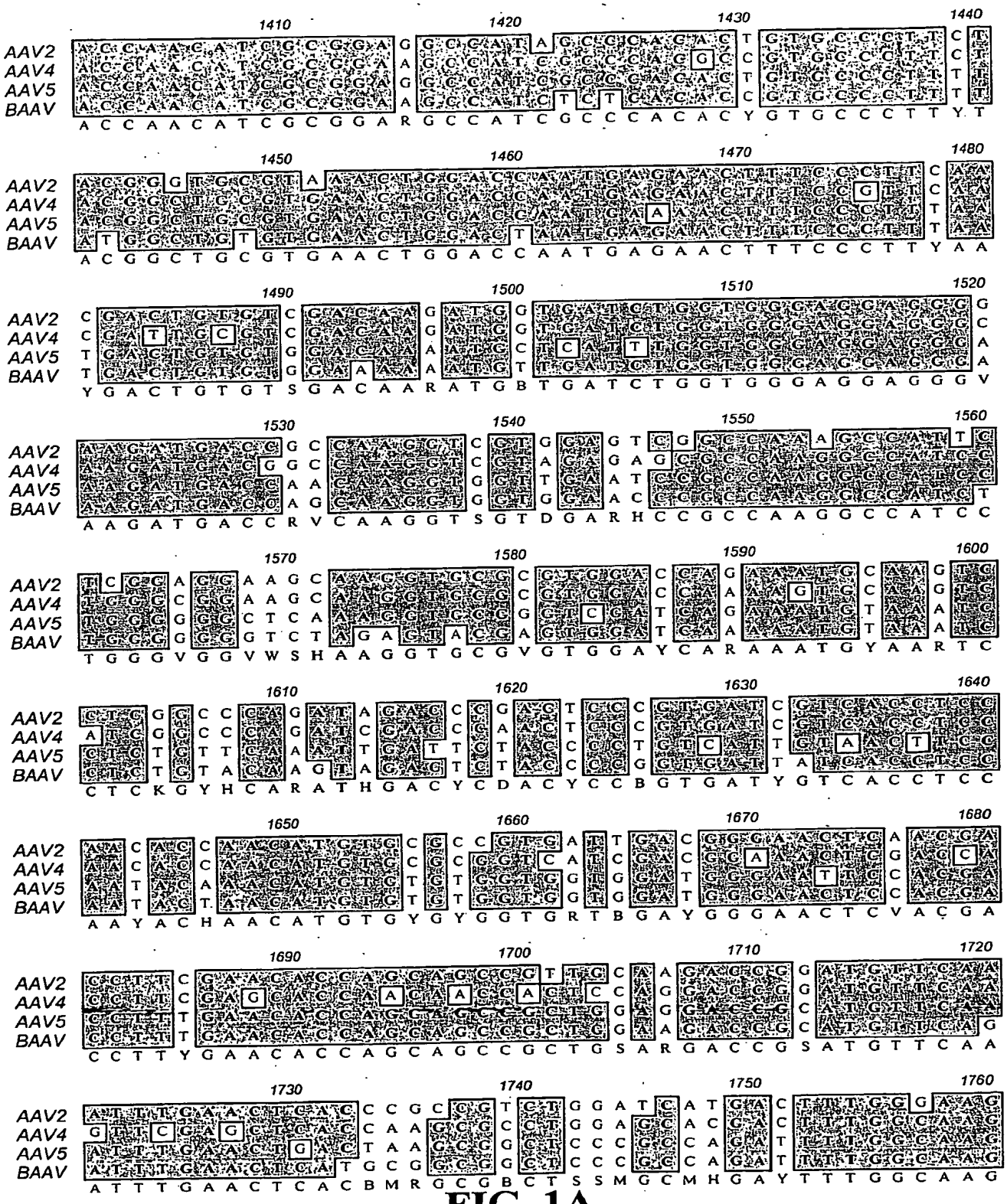

1410
1420
1430
1440
AAV2
AAV4
AAV5
BAAV
1450
1460
1470
1480
1490
1500
1510
1520
1530
1540
1550
1560
1570
1580
1590
1600
1610
1620
1630
1640
1650
1660
1670
1680
1690
1700
1710
1720
1730
1740
1750
1760

FIG. 1A

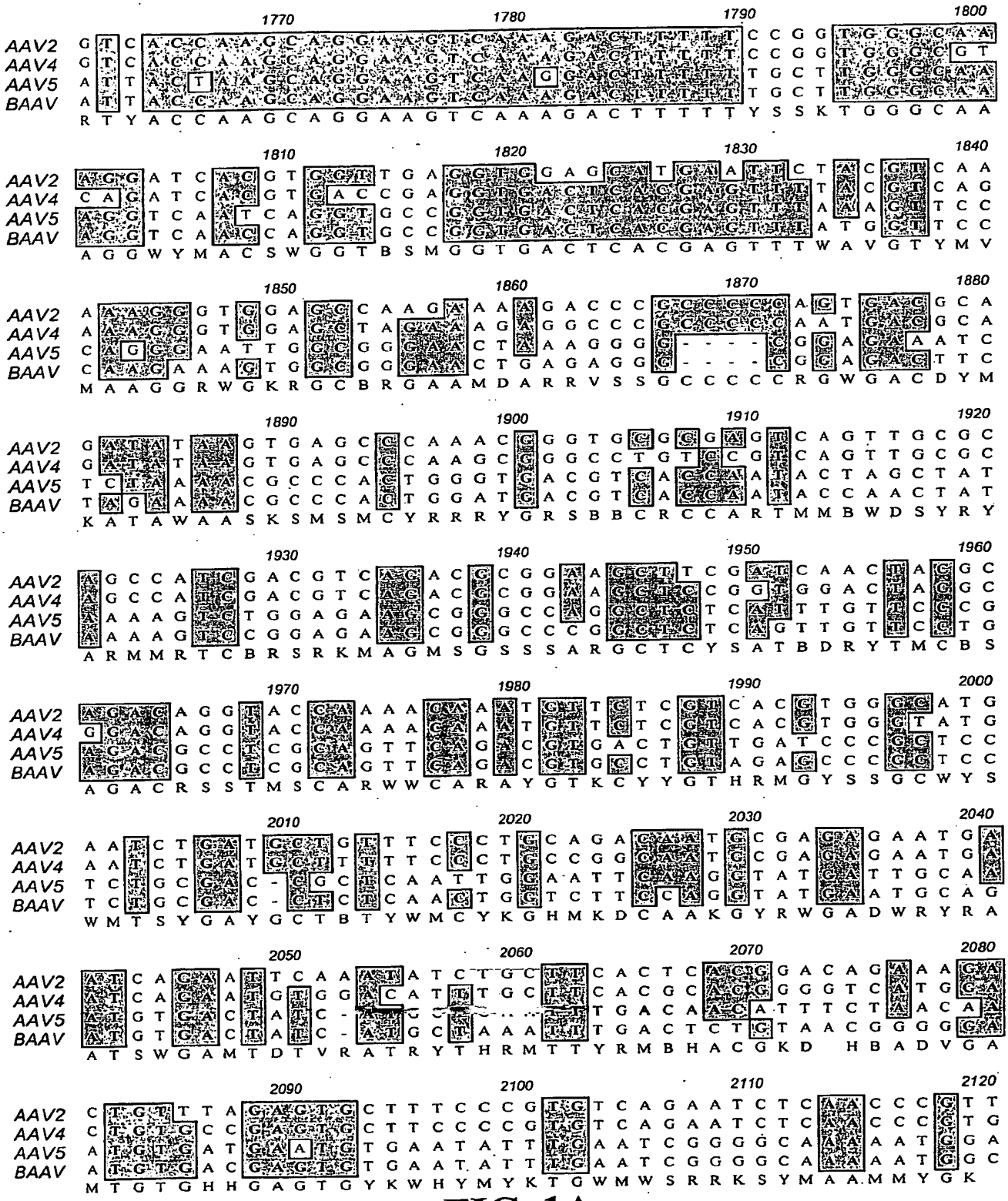

1770
1780
1790
1800
AAV2
AAV4
AAV5
BAAV
1810
1820
1830
1840
1850
1860
1870
1880
1890
1900
1910
1920
1930
1940
1950
1960
1970
1980
1990
2000
2010
2020
2030
2040
2050
2060
2070
2080
2090
2100
2110
2120

FIG. 1A

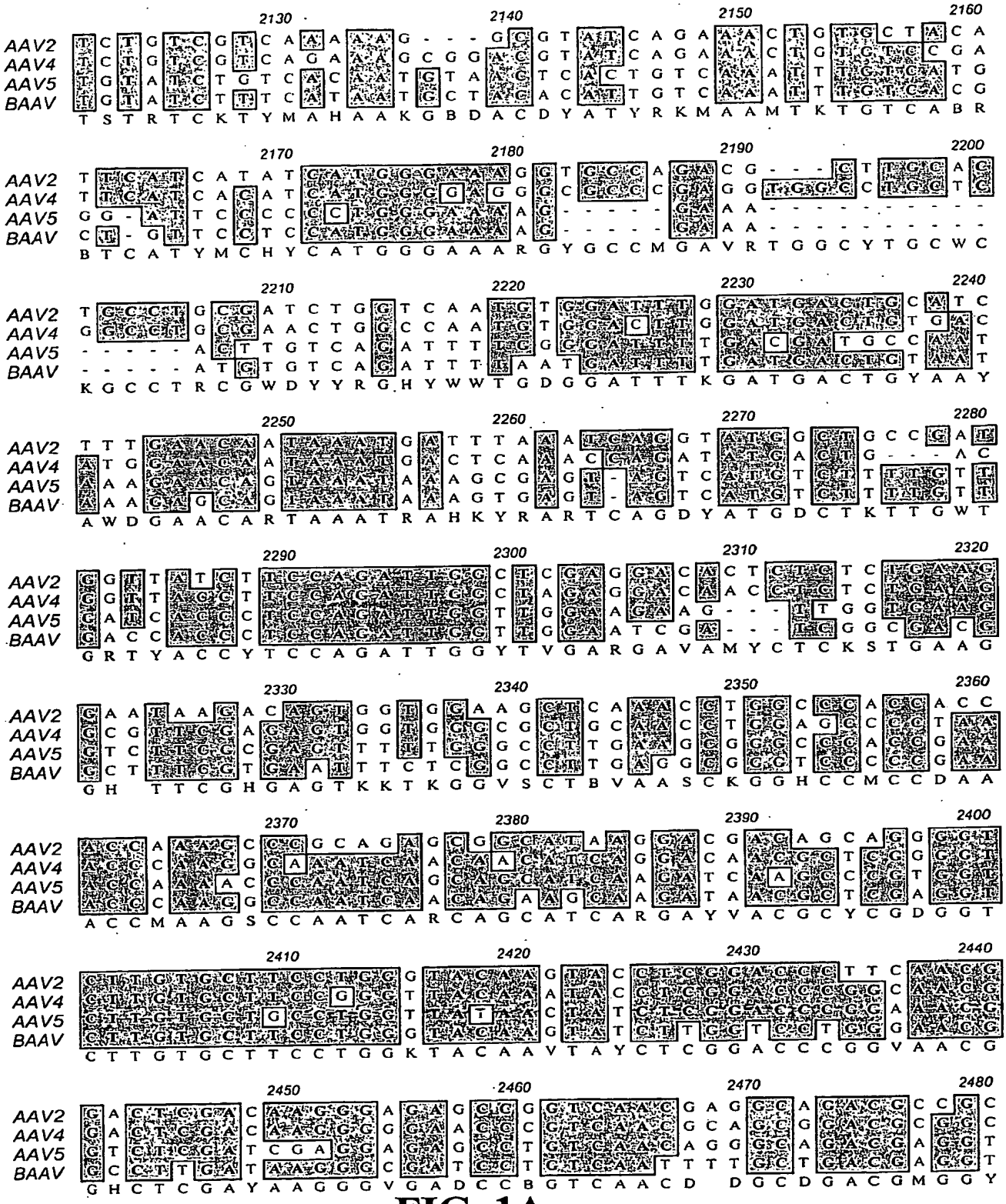

2130
2140
2150
2160
AAV2
AAV4
AAV5
BAAV
T S T R T C K T Y M A H A A K G B D A C D Y A T Y R K M A A M T K T G T C A B R
2170
2180
2190
2200
B T C A T Y M C H Y C A T G G G A A A R G Y G C C M G A V R T G G C Y T G C W C
2210
2220
2230
2240
K G C C T R C G W D Y Y R G H Y W W T G D G G A T T T K G A T G A C T G Y A A Y
2250
2260
2270
2280
A W D G A A C A R T A A A T R A H K Y R A R T C A G D Y A T G D C T K T T G W T
2290
2300
2310
2320
G R T Y A C C Y T C C A G A T T G G Y T V G A R G A V A M Y C T C K S T G A A G
2330
2340
2350
2360
G H T T C G H G A G T K K T K G G V S C T B V A A S C K G G H C C M C C D A A
2370
2380
2390
2400
A C C M A A G S C C A A T C A R C A G C A T C A R G A Y V A C G C Y C G D G G T
2410
2420
2430
2440
C T T G T G C T T C C T G G K T A C A A V T A Y C T C G G A C C C G G V A A C G
2450
2460
2470
2480
G H C T C G A Y A A G G G V G A D C C B G T C A A C D D G C D G A C G M G G Y

FIG. 1A

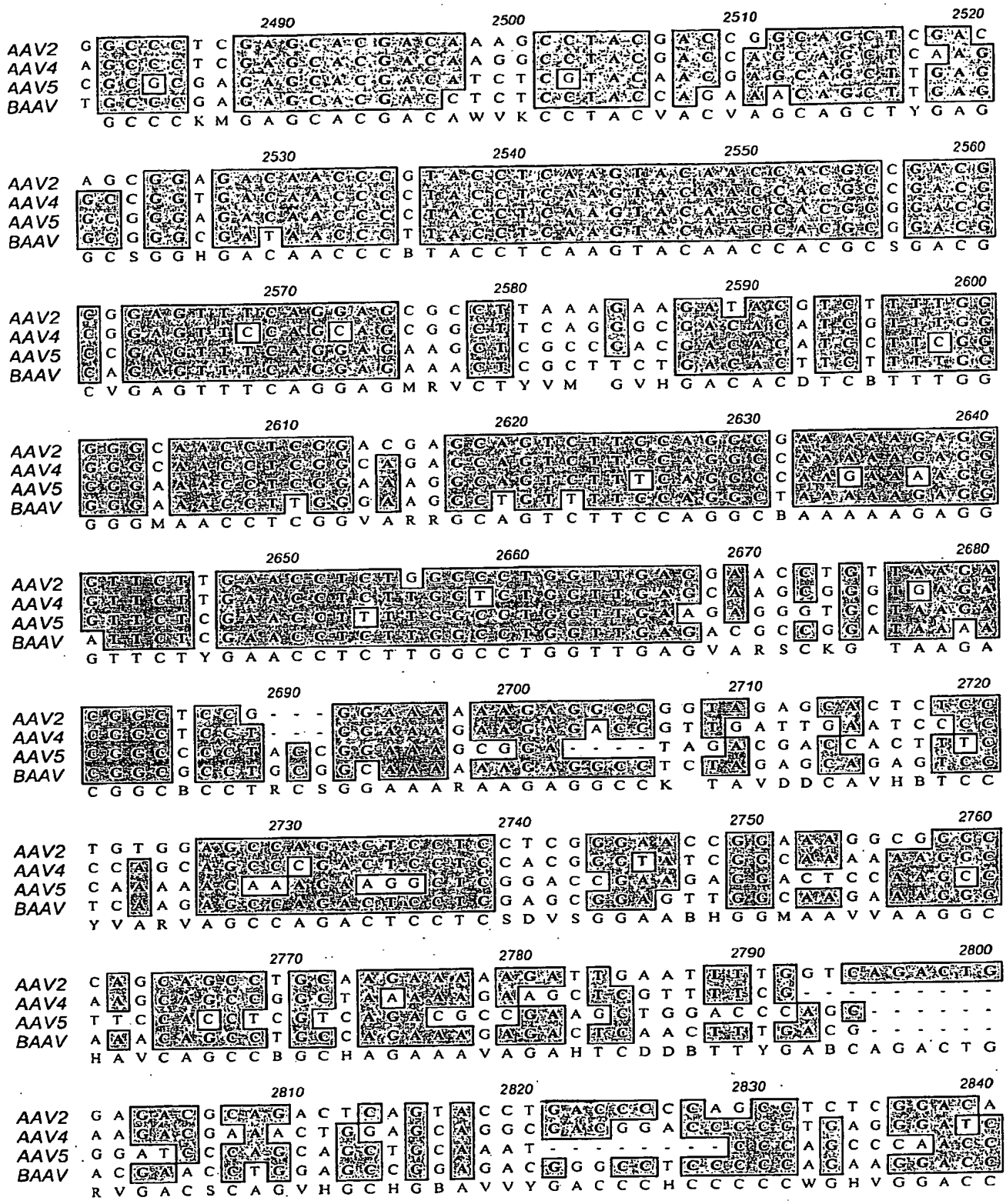

2490
2500
2510
2520
AAV2
AAV4
AAV5
BAAV
2530
2540
2550
2560
2570
2580
2590
2600
2610
2620
2630
2640
2650
2660
2670
2680
2690
2700
2710
2720
2730
2740
2750
2760
2770
2780
2790
2800
2810
2820
2830
2840

FIG. 1A

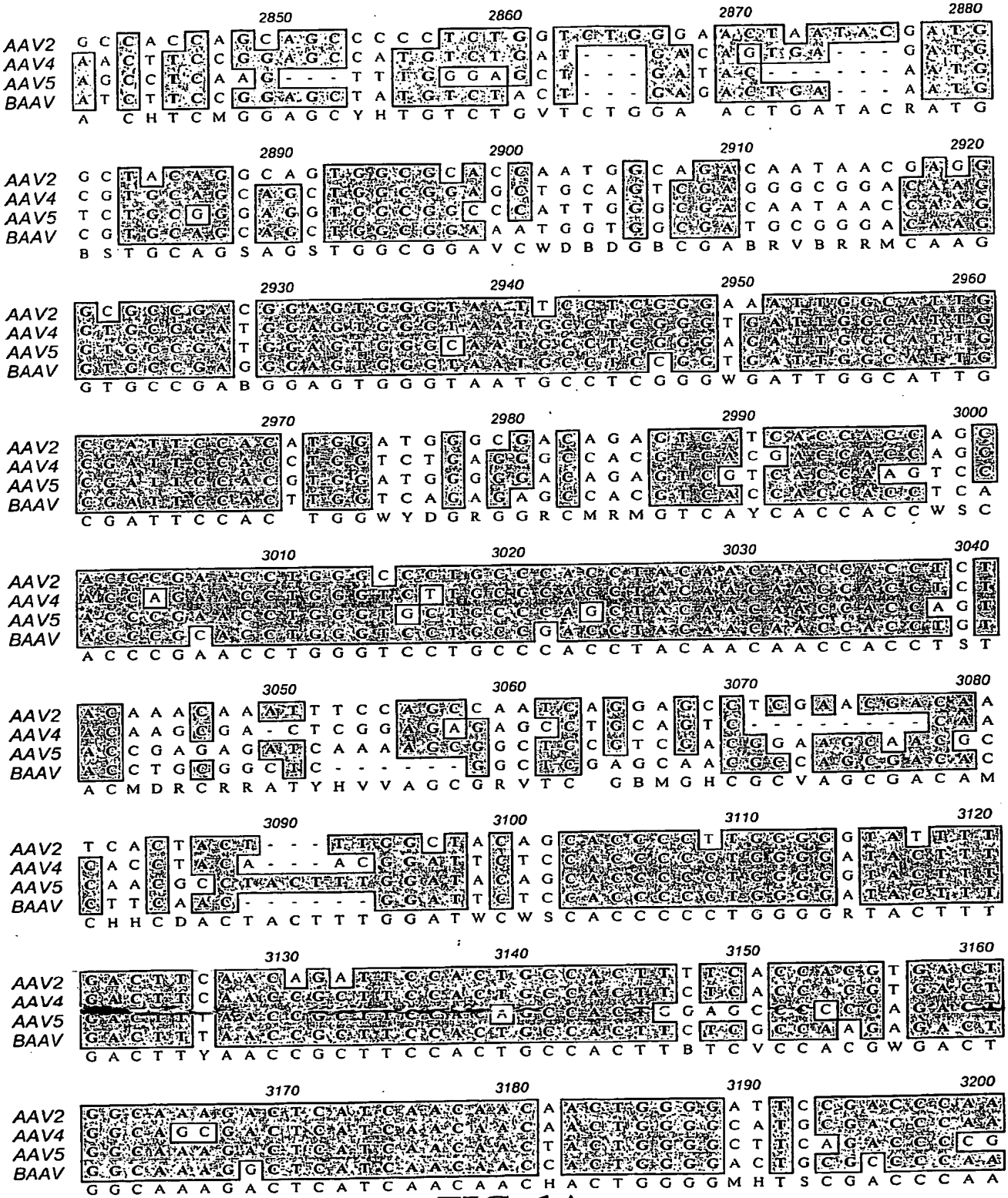
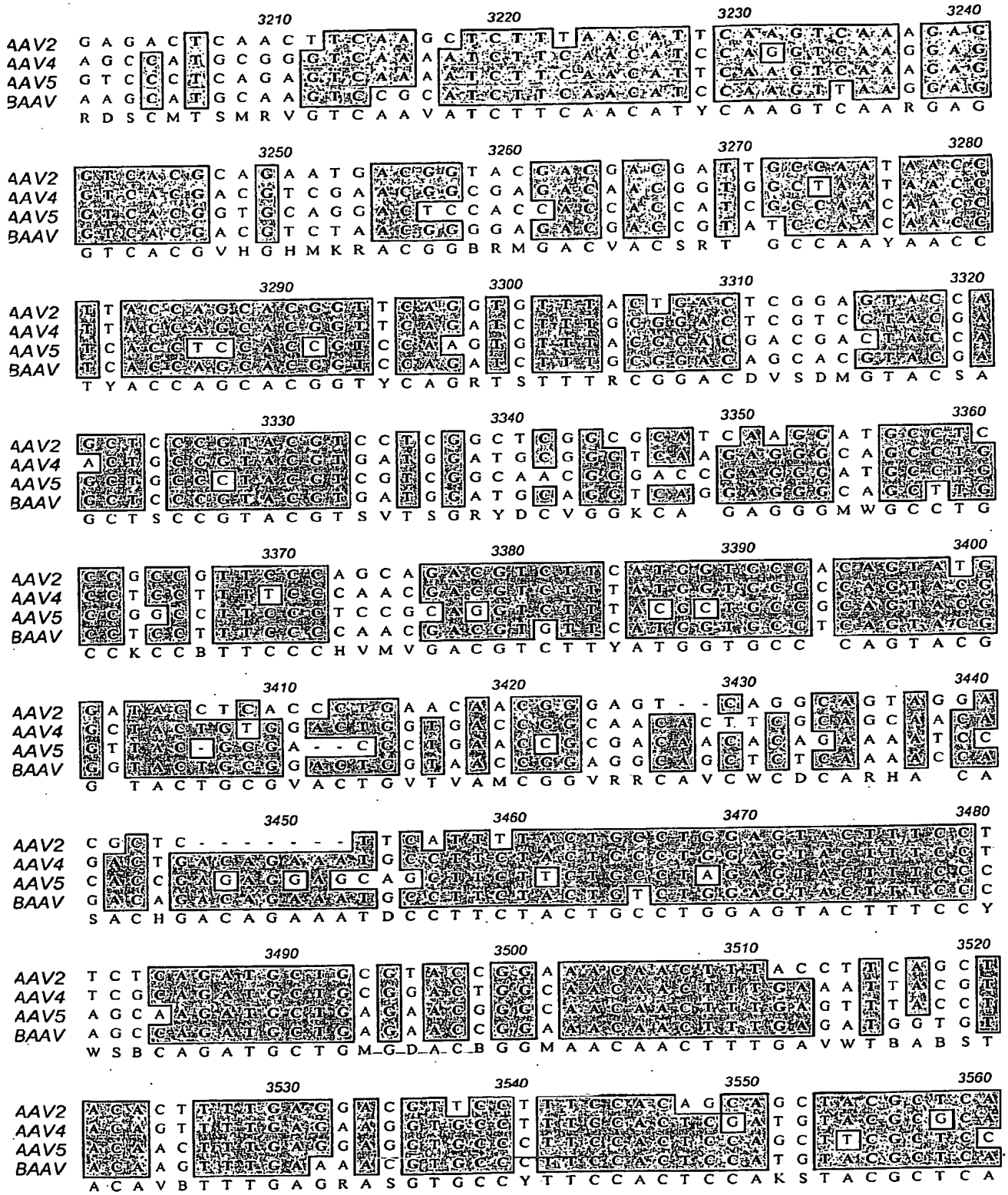

2850
2860
2870
2880
AAV2
AAV4
AAV5
BAAV
2890
2900
2910
2920
2930
2940
2950
2960
2970
2980
2990
3000
3010
3020
3030
3040
3050
3060
3070
3080
3090
3100
3110
3120
3130
3140
3150
3160
3170
3180
3190
3200

FIG. 1A


3210
3220
3230
3240
3250
3260
3270
3280
3290
3300
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3320
3330
3340
3350
3360
3370
3380
3390
3400
3410
3420
3430
3440
3450
3460
3470
3480
3490
3500
3510
3520
3530
3540
3550
3560
AAV2
AAV4
AAV5
BAAV

FIG. 1A

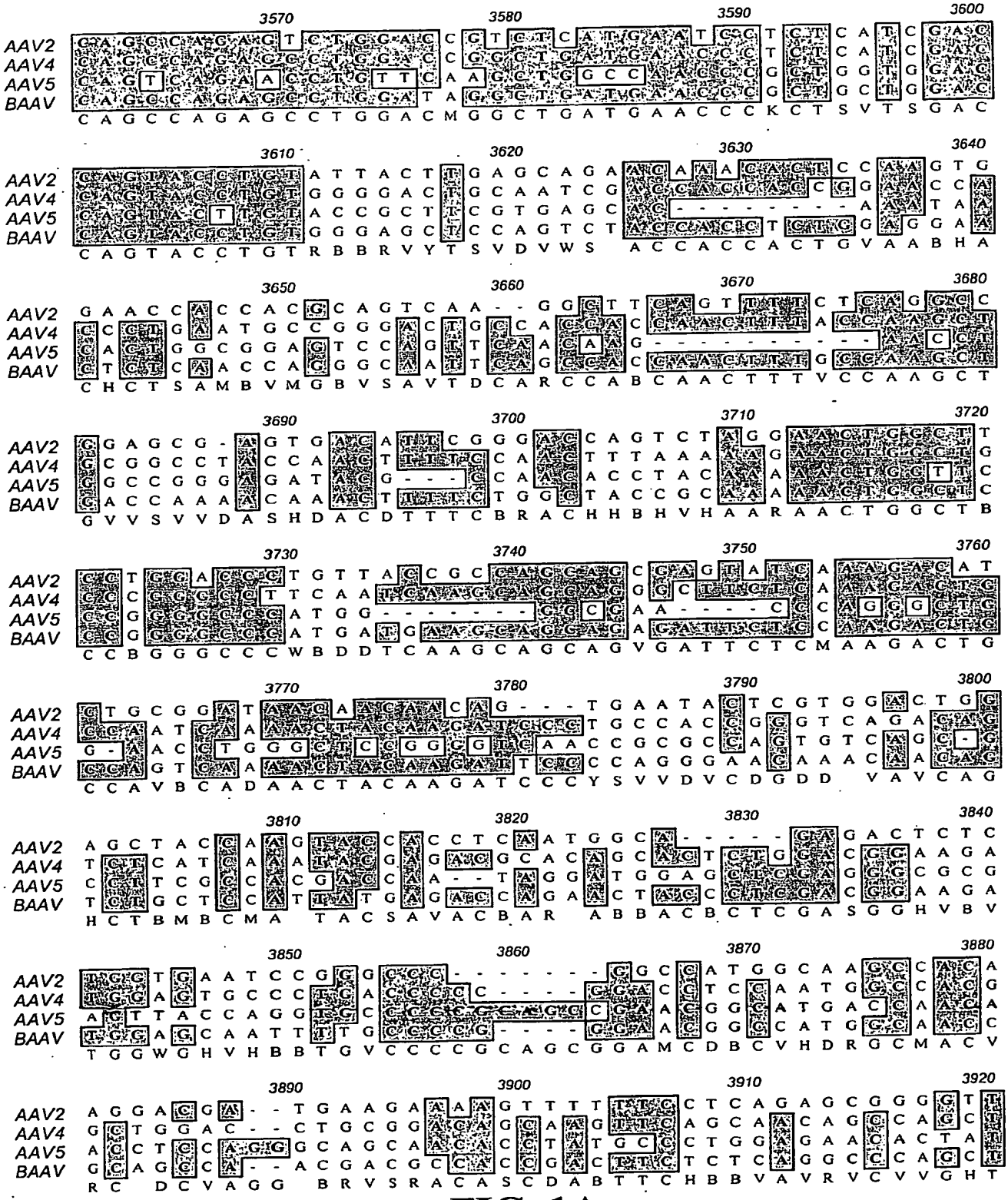

3570
3580
3590
3600
AAV2
AAV4
AAV5
BAAV
CAGCCAGAGCCTGGACMGGCTGATGAACCCKCTSVTSGAC
3610
3620
3630
3640
CAGTACCTGTRBBRVYTSVDVWS ACCACCACTGVAABHA
3650
3660
3670
3680
CHCTSAMBVMGBVSAVTDCARCCABCAACTTTVCCAAGCT
3690
3700
3710
3720
GVVSVVDASHDACDTTTCBRACHHBHVHAARAACTGGCTB
3730
3740
3750
3760
CCBGGGCCCWBDDTCAAGCAGCAGVGATTCTCMAAGACTG
3770
3780
3790
3800
CCAVBCADAACTACAAGATCCCYSVVDVCDGDD VAVCAG
3810
3820
3830
3840
HCTBMBCMA TACSAVACBAR ABBACBCTCGASGGHVBV
3850
3860
3870
3880
TGGWGHVHBBTGVCCCCGCAGCGGAMCDBCVHDRGCMACV
3890
3900
3910
3920
RC DCVAGG BRVSRACASCDABTTCHBBVAVRVCVVGHT

FIG. 1A

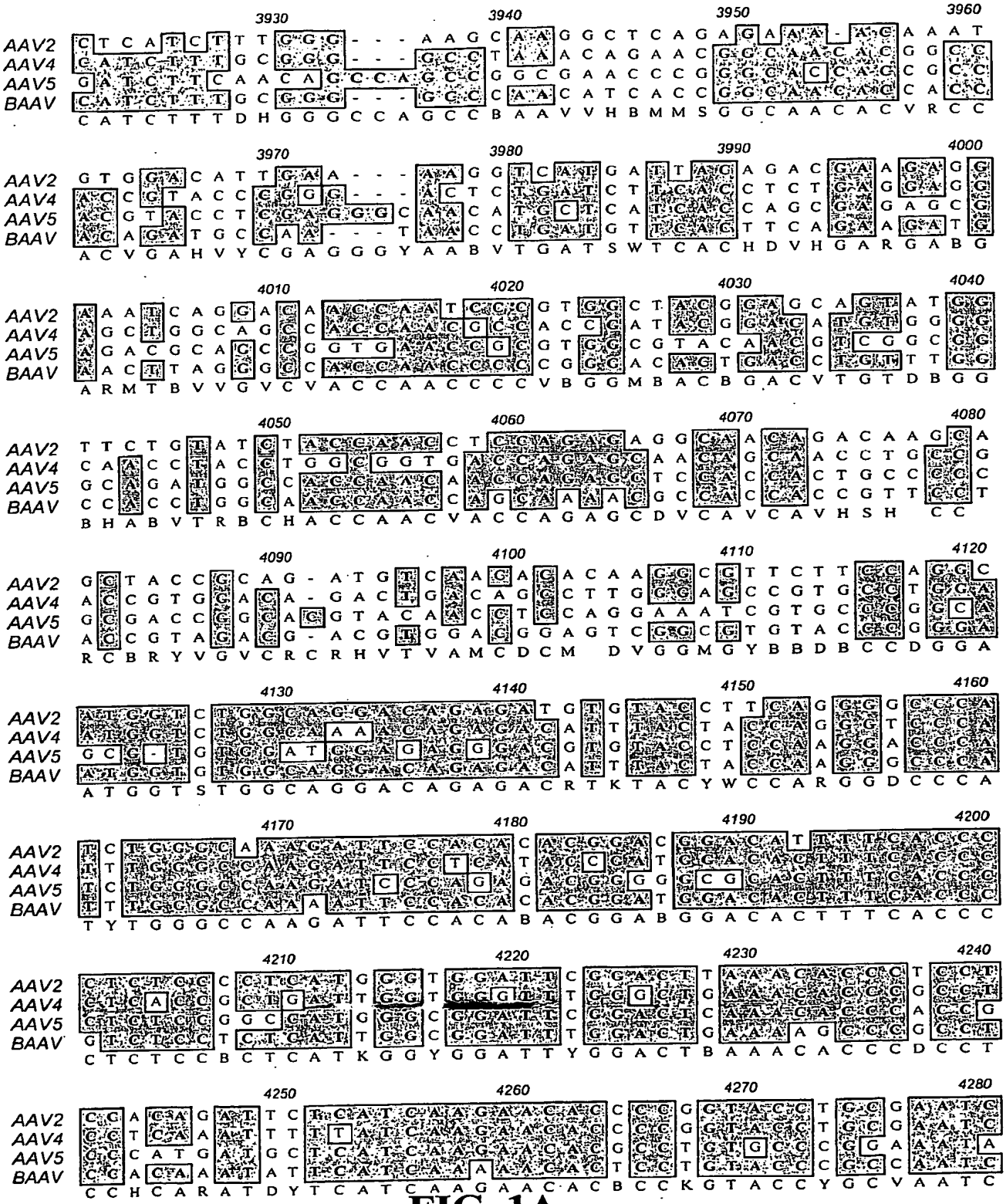
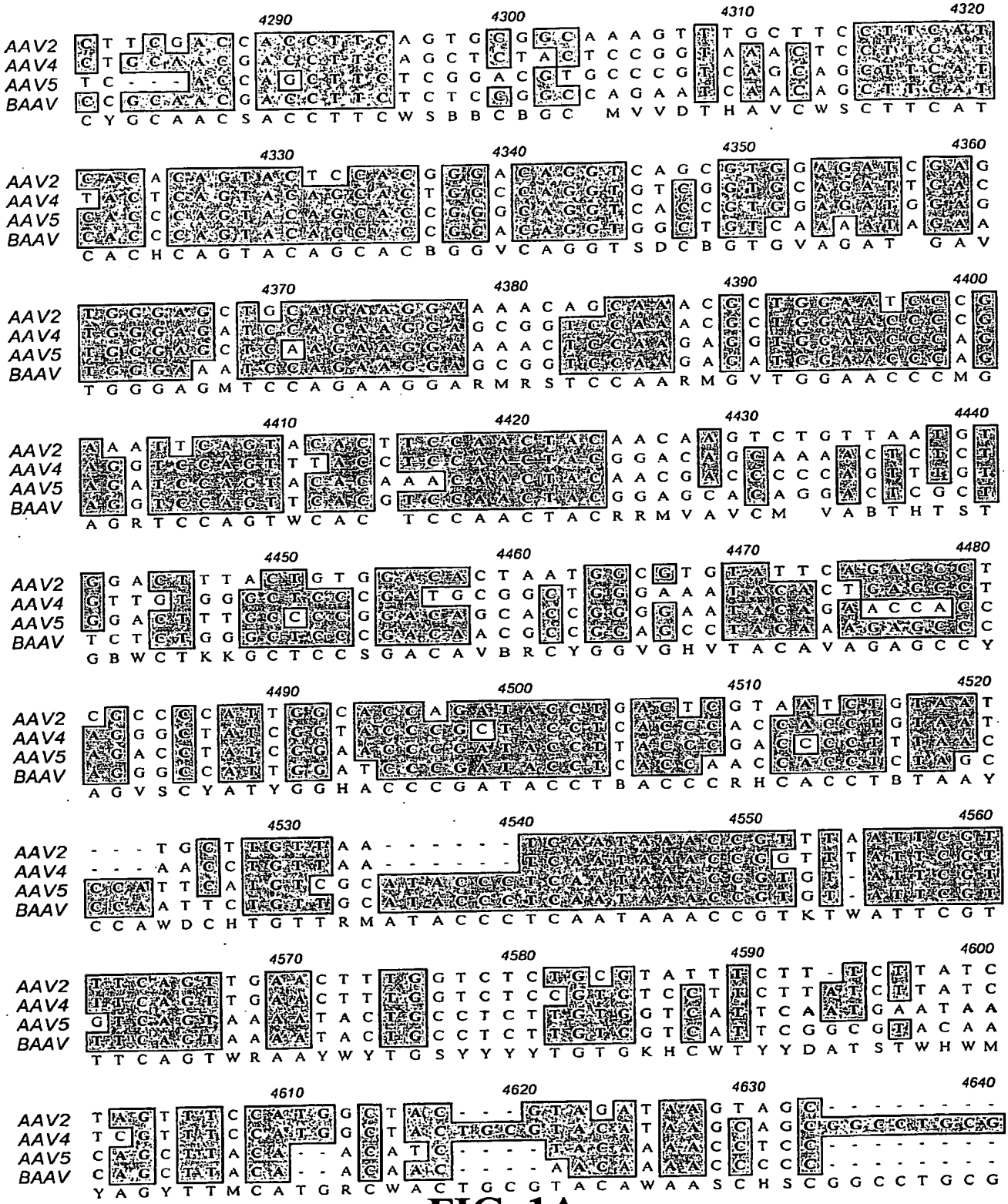

3930
3940
3950
3960
AAV2
AAV4
AAV5
BAAV
3970
3980
3990
4000
4010
4020
4030
4040
4050
4060
4070
4080
4090
4100
4110
4120
4130
4140
4150
4160
4170
4180
4190
4200
4210
4220
4230
4240
4250
4260
4270
4280

FIG. 1A


4290
4300
4310
4320
AAV2
AAV4
AAV5
BAAV
4330
4340
4350
4360
4370
4380
4390
4400
4410
4420
4430
4440
4450
4460
4470
4480
4490
4500
4510
4520
4530
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4550
4560
4570
4580
4590
4600
4610
4620
4630
4640

FIG. 1A

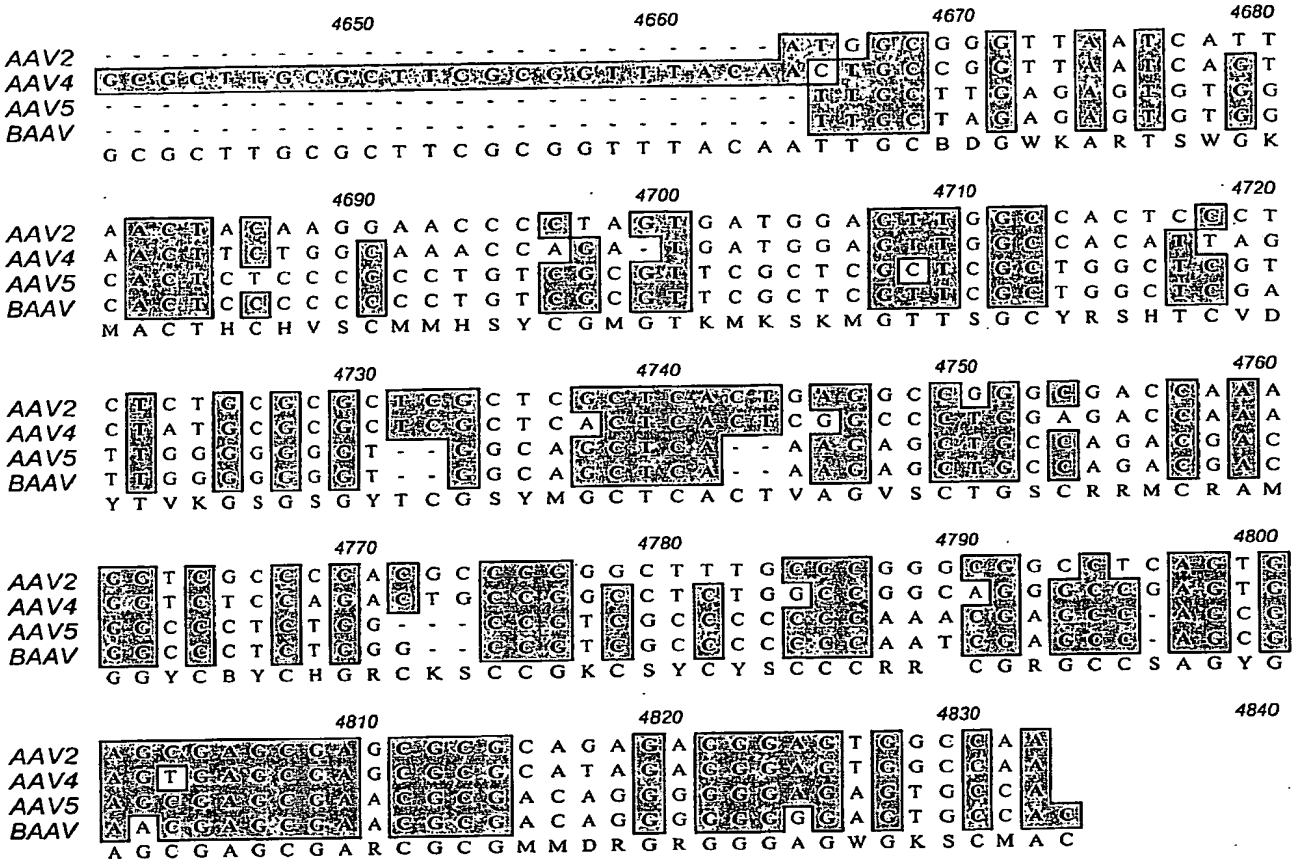

4650
4660
4670
4680
AAV2
AAV4
AAV5
BAAV
G C G C T T G C G C T T C G C G G T T T A C A A T T G C B D G W K A R T S W G K
4690
4700
4710
4720
M A C T H C H V S C M M H S Y C G M G T K M K S K M G T T S G C Y R S H T C V D
4730
4740
4750
4760
Y T V K G S G S G Y T C G S Y M G C T C A C T V A G V S C T G S C R R M C R A M
4770
4780
4790
4800
G G Y C B Y C H G R C K S C C G K C S Y C Y S C C C R R C G R G C C S A G Y G
4810
4820
4830
4840
A G C G A G C G A R C G C G M M D R G R G G G A G W G K S C M A C

FIG. 1A

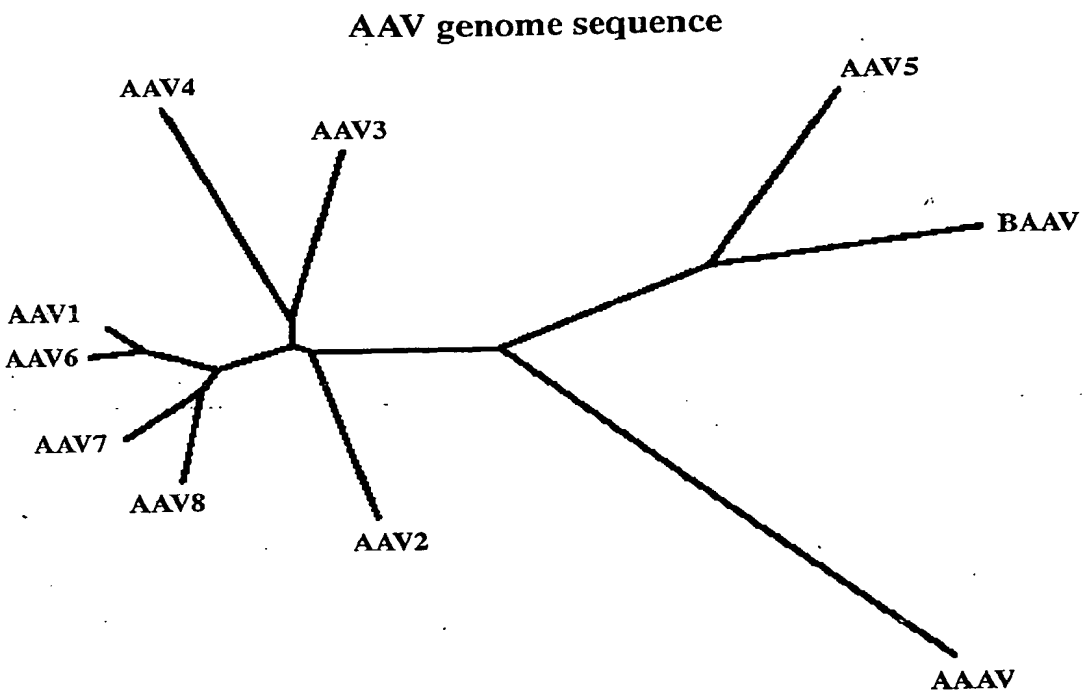

AAV genome sequence
AAV4
AAV3
AAV5
BAAV
AAV1
AAV6
AAV7
AAV8
AAV2
AAAV

FIG. 1B

TRS
Rep binding site
ACAAAACCCCCTTGCTAGAG
TGTTTTGGGGGAACGATCTC
AGTGTGG
TCACACC
CACTCCCCCCCCCTGTCGC
GTGAGGGGGGGGACAGCG
GTTCGCTCGTTCGCTGGCTCG
CAAGCGAGCAAGCGACCGAGC
ATTGGGGGGG
TAACCCCCCC

FIG. 2

Rep78

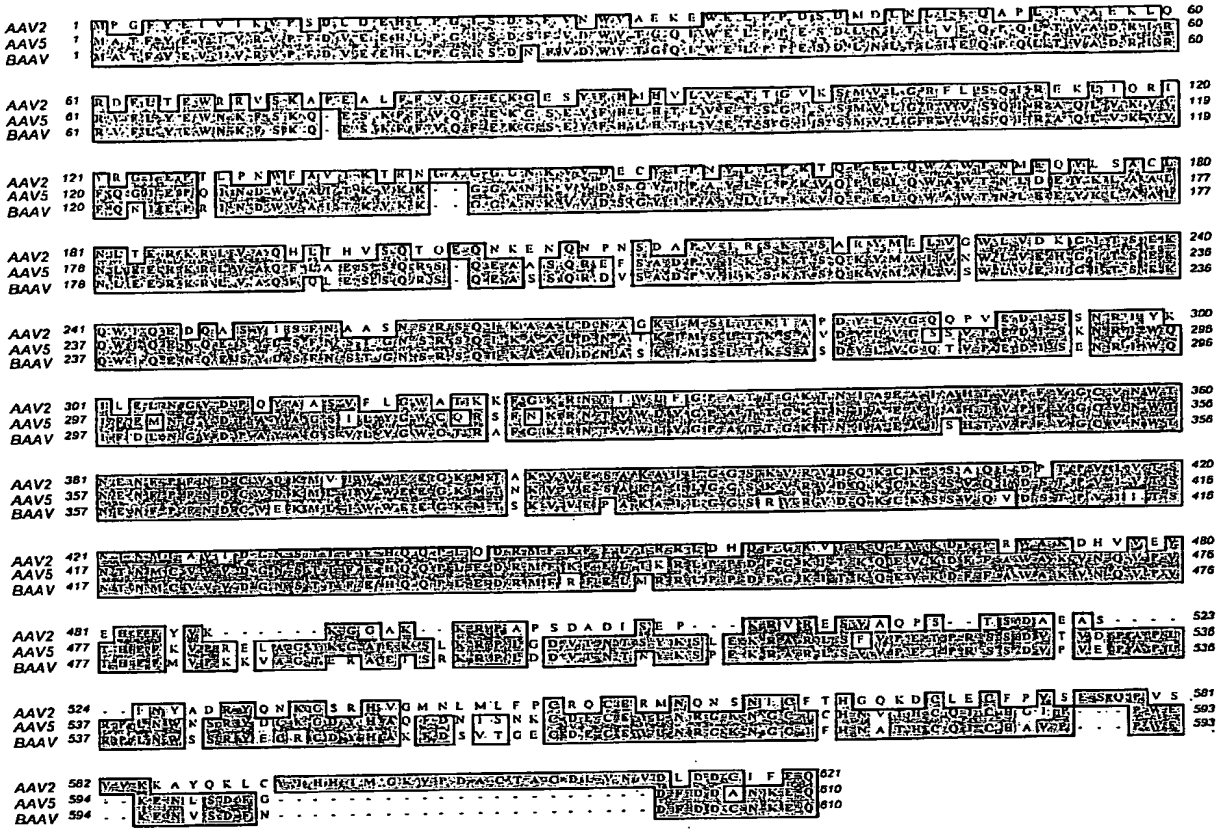


FIG. 3A

VP1

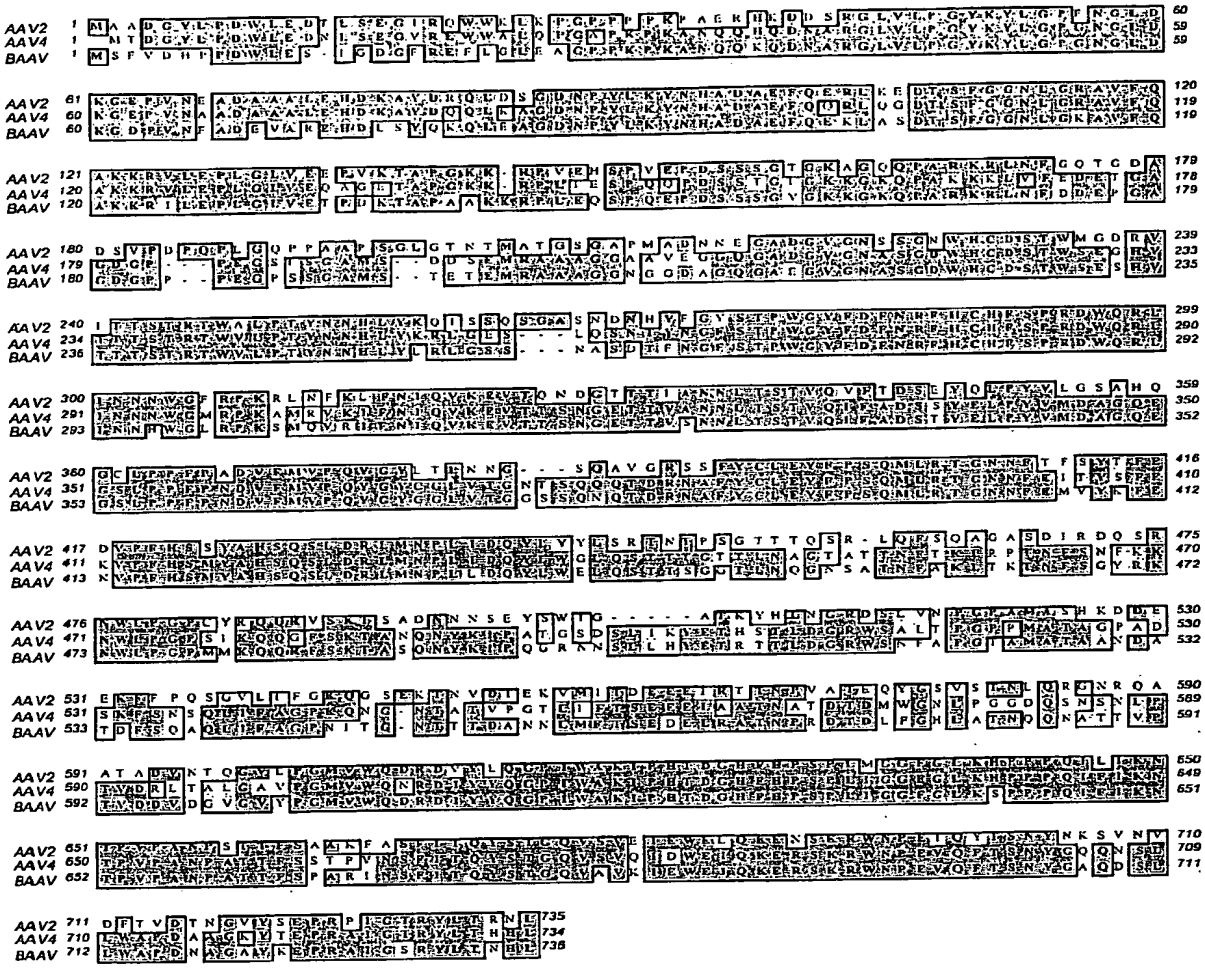


FIG. 3B

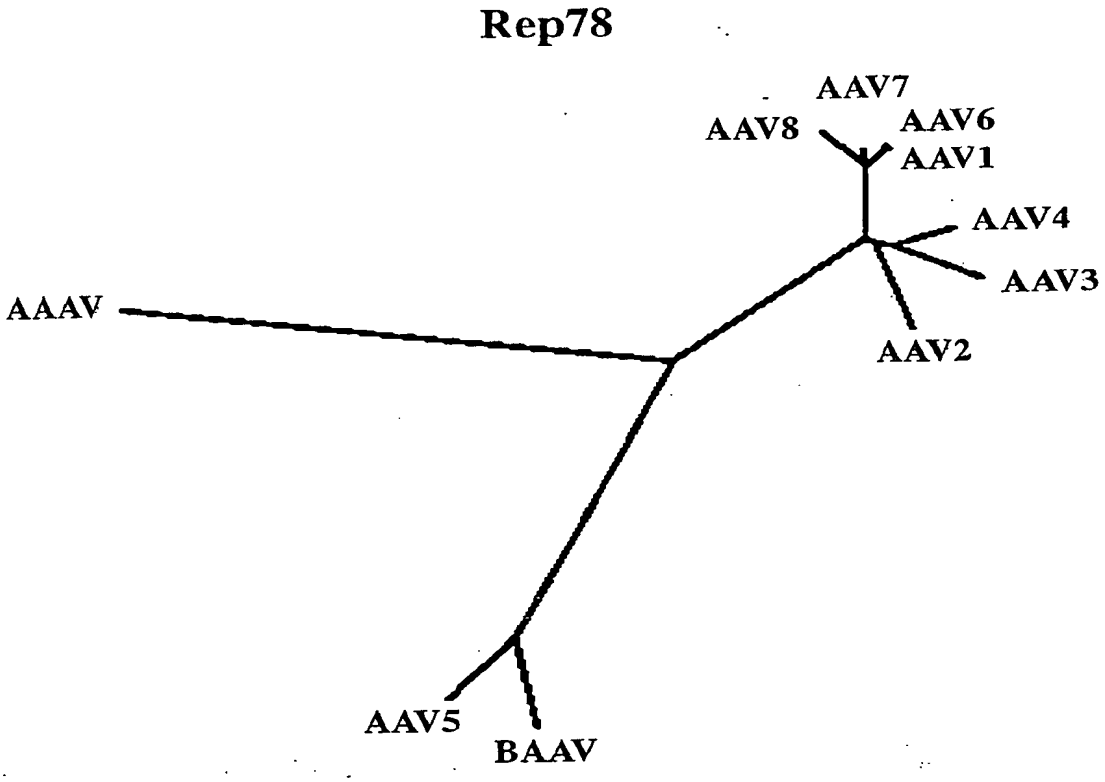


FIG. 3C

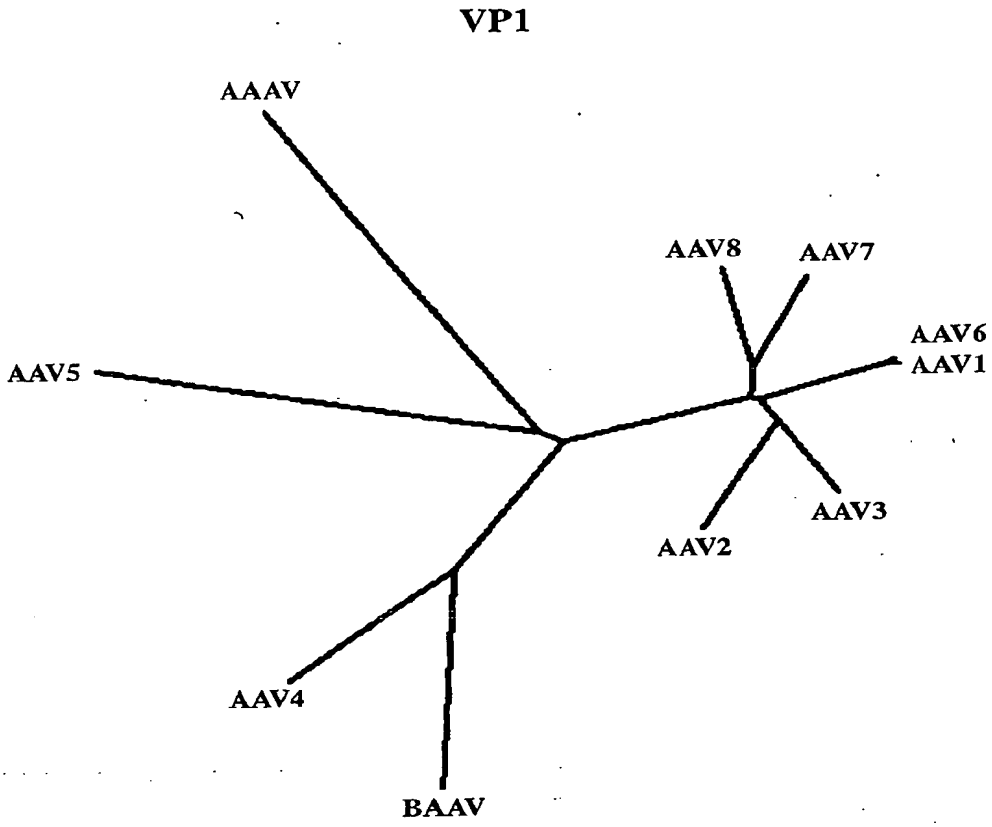
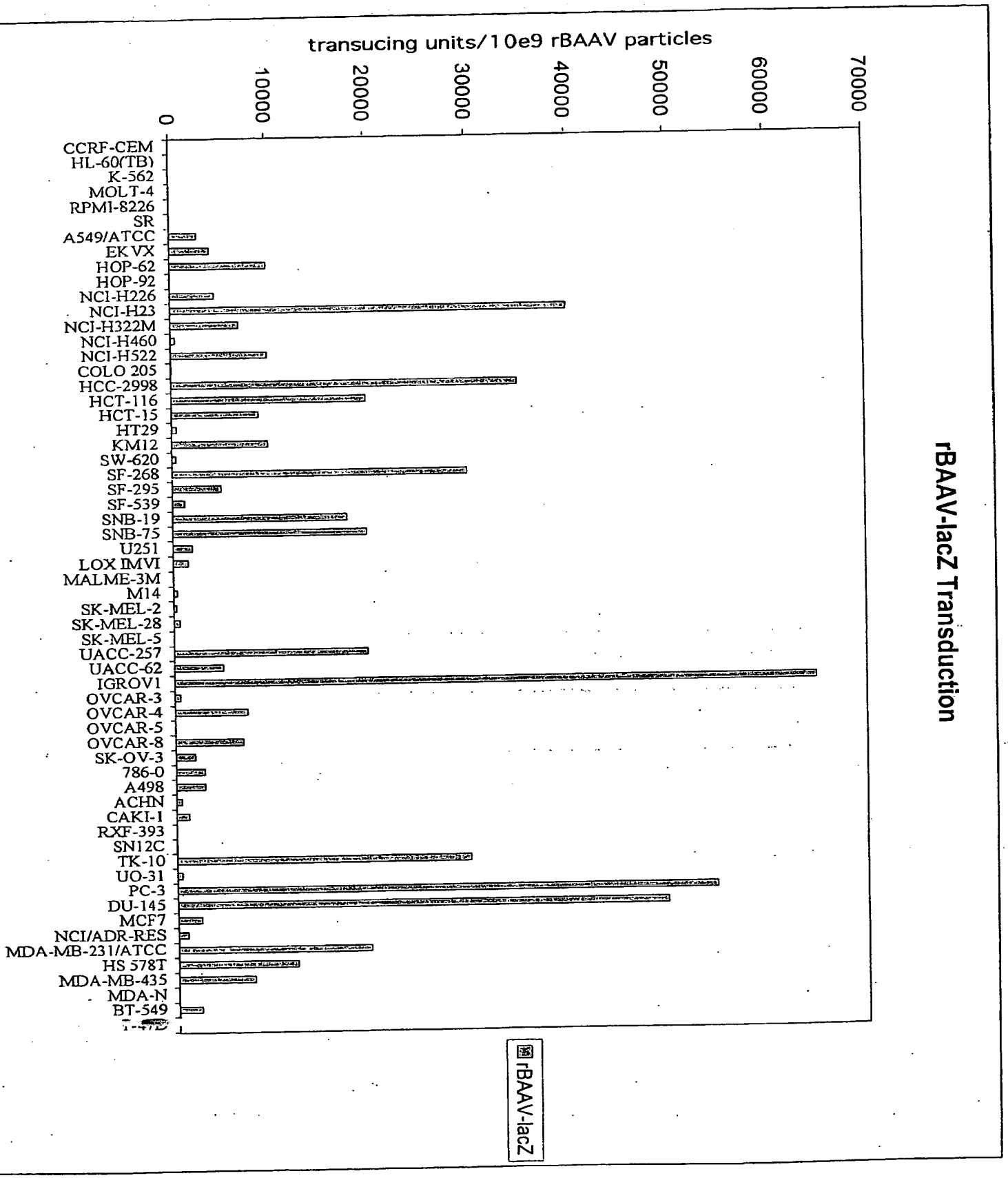


FIG. 3D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0
10000
20000
30000
40000
50000
60000
70000
CCRF-CEM
HL-60(TB)
K-562
MOLT-4
RPMI-8226
SR
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522
COLO 205
HCC-2998
HCT-116
HCT-15
HT29
KM12
SW-620
SF-268
SF-295
SF-539
SNB-19
SNB-75
U251
LOX IMVI
MALME-3M
M14
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3
786-0
A498
ACHN
CAKI-1
RXF-393
SN12C
TK-10
UO-31
PC-3
DU-145
MCF7
NCI/ADR-RES
MDA-MB-231/ATCC
HS 578T
MDA-MB-435
MDA-N
BT-549
rBAAV-lacZ
rBAAV-lacZ Transduction

FIG. 4

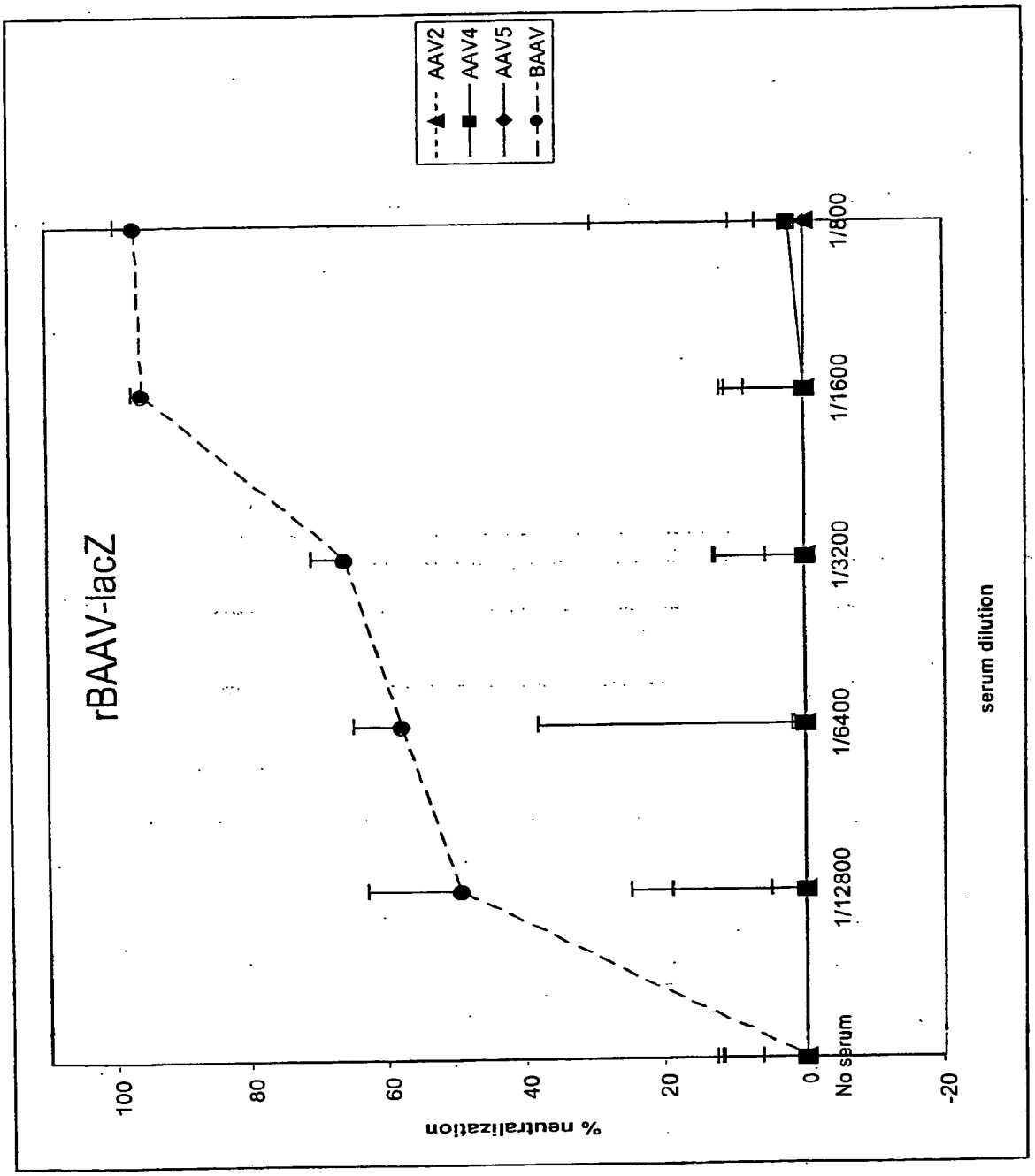
AAV2
AAV4
AAV5
BAAV
rBAAV-lacZ
% neutralization
100
80
60
40
20
0
-20
No serum
1/12800
1/6400
1/3200
1/1600
1/800
serum dilution

FIG. 5A

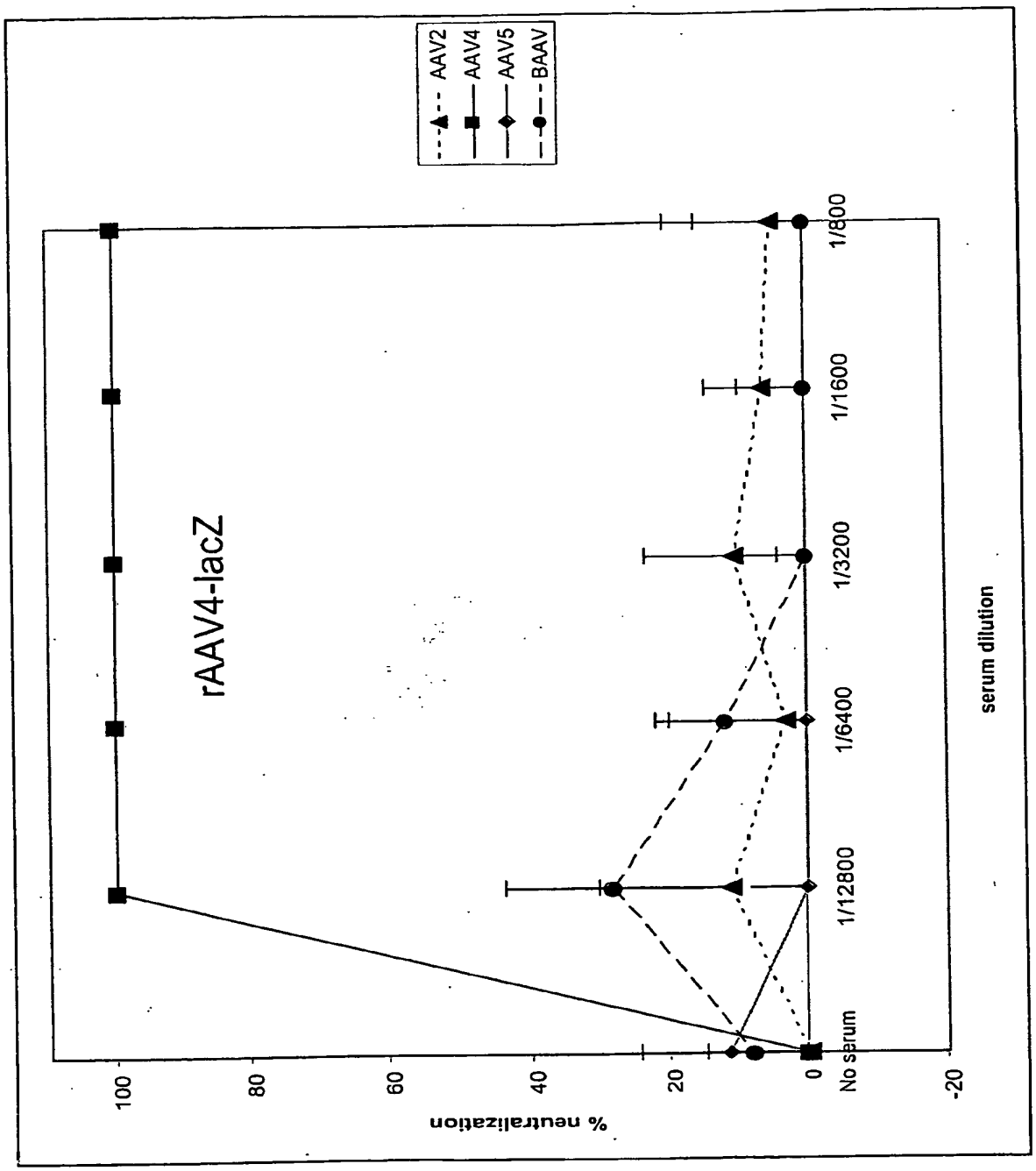
AAV2
AAV4
AAV5
BAAV
rAAV4-lacZ
% neutralization
100
80
60
40
20
0
-20
No serum
1/12800
1/6400
1/3200
1/1600
1/800
serum dilution

FIG. 5B

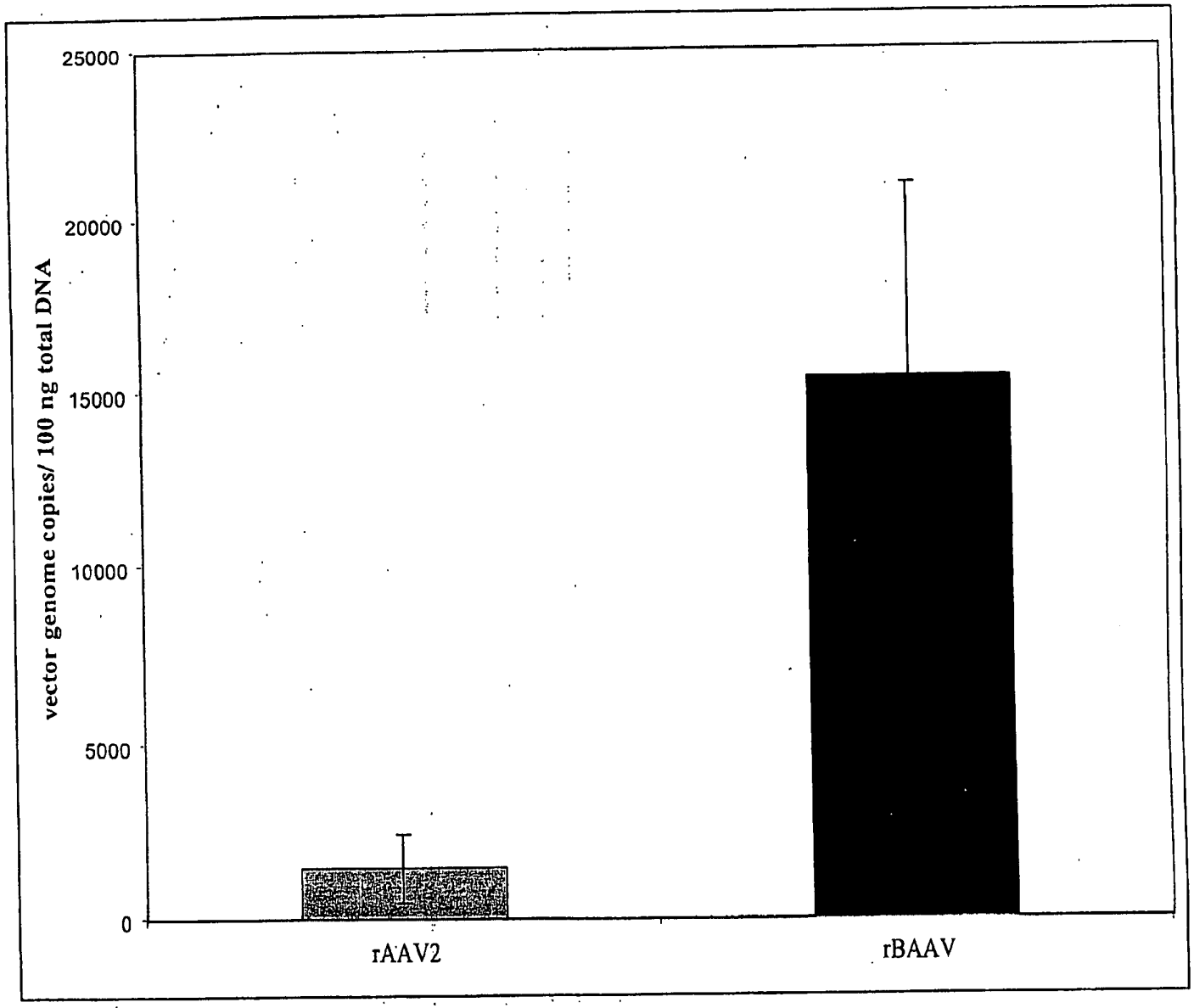
25000
20000
15000
10000
5000
0
vector genome copies/ 100 ng total DNA
rAAV2
rBAAV

FIG. 6A

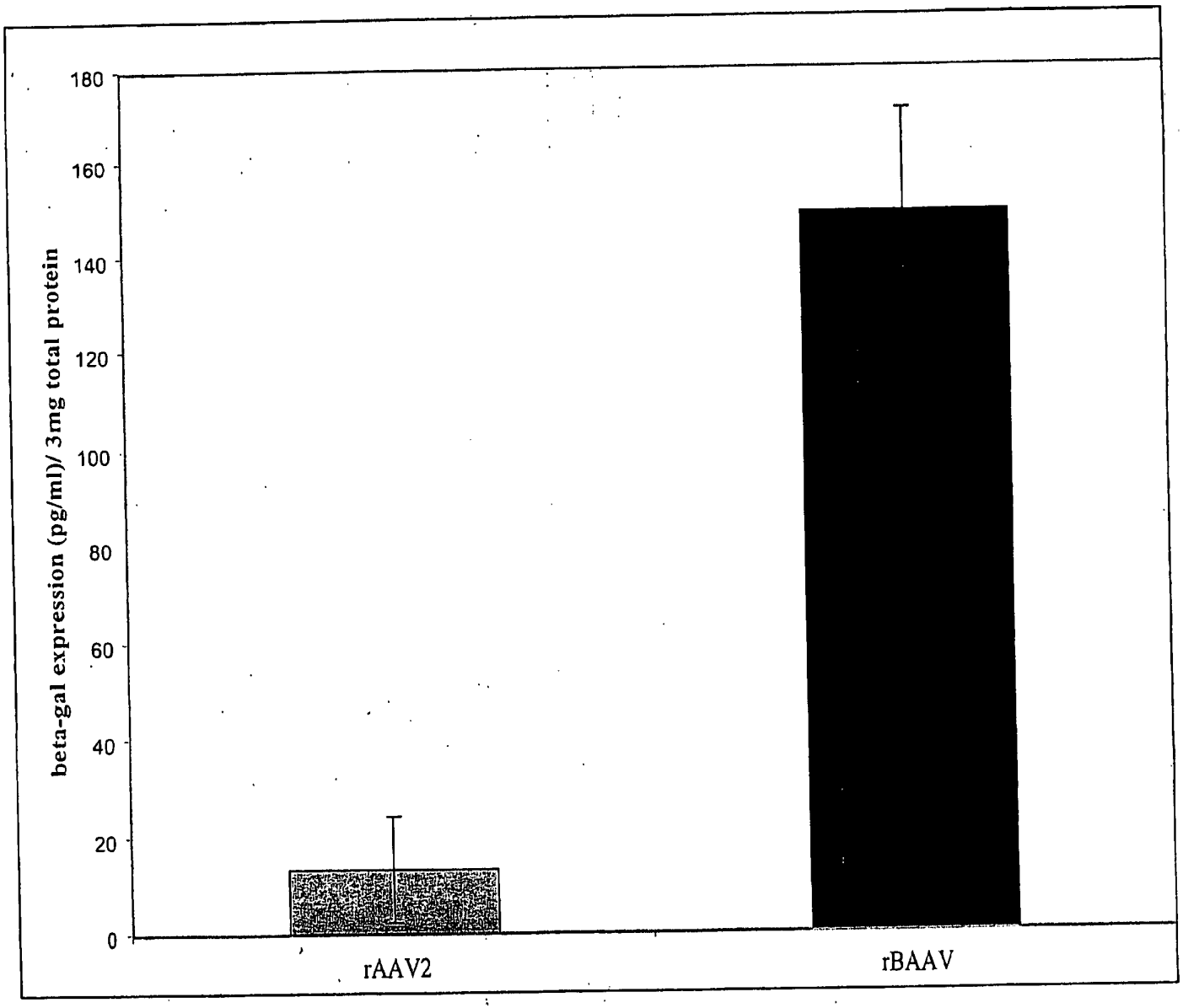
beta-gal expression (pg/ml)/ 3mg total protein
180
160
140
120
100
80
60
40
20
0
rAAV2
rBAAV

FIG. 6B

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